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Stéphanie Pereira Oliveira **Study of the role of ceramide in acetate-induced cell death in colorectal carcinoma cell lines**

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Dissertação de Mestrado
Mestrado em Genética Molecular

Trabalho realizado sob a orientação da
Professora Ana Arminda Lopes Preto Almeida
e da
Professora Maria Manuela Sansonetty Gonçalves Côrte-Real

Outubro de 2012

Declaração

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DESIGNAÇÃO DO MESTRADO:

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"Vous savez ce que c'est que la recherche : on part sur une question et on trouve en cours de route des faits qui vous en posent une autre." – Philippe Meyer

A conclusão deste projeto representa, não só mais um ciclo de estudos, mas também o início de uma nova fase. Mais do que uma simples experiência no laboratório, este projeto foi uma grande ajuda em termos de crescimento pessoal. Obrigado a todos os que, de alguma forma, me ajudaram a concretizá-lo!

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Abstract

Colorectal carcinoma (CRC) has one of the highest incidences and mortality on the modern society, but a growing impact on less developed and economically transitioning nations. Prevention, surgery and radio/chemotherapy are the principal approaches to overcome the disease. Finding new efficient/specific treatments in CRC, better tolerated by patients is mandatory.

News strategies to be used against colorectal cancer emerged when it was found that certain strains of *Propionibacterium* could protect against CRC. Those bacteria, living in the human intestine, produce short-chain fatty acids (SCFAs), namely acetate, butyrate and propionate that are able to induce apoptosis specifically on CRC cells. The mechanisms behind these occurrences are not fully understood. Ceramide is an important signalling molecule, vital in cellular processes and may play a role in regulation of apoptosis in CRC cells. Previous research executed by our laboratory showed that acetate-induced apoptosis in CRC cell lines and lysosome membrane permeabilization (LMP) with cathepsin D release. The fact that ceramide promotes LMP and activates cathepsin D, led us to hypothesize that ceramide might be generated in response to acetate and thus mediate apoptosis induced by this lethal stimulus, in CRC cells.

The main objective of the project was to assess the involvement of ceramide in acetate-induced apoptosis on CRC derived cell lines (HCT-15 and RKO). In order to reach that goal, cells were treated with acetate and/or inhibitors of the ceramide biosynthesis pathways (GW4869, fumonisin B_1 and myriocin) and cellular viability was analysed by MTT assay.

The inhibition of the three major pathways of ceramide generation (*de novo*, salvage and sphingomyelinase pathways) did not change significantly the cellular viability, on both cell lines. Indeed the expected increase in cellular viability did not occur when cells were treated with acetate and one or more inhibitors of ceramide synthesis pathways. Further studies are necessary to confirm the results obtained, namely determining ceramide cellular levels, and the activity of enzymes involved in ceramide synthesis treated with acetate and/or ceramide inhibitors.

Summing up, the results obtained so far suggest that acetate-induced cell death in HCT-15 and RKO CRC cells might not be mediated by ceramide.

Resumo

O carcinoma colo-rectal (CCR) tem uma das maiores incidências e mortalidade na sociedade moderna, mas um impacto crescente em países menos desenvolvidos e em vias de desenvolvimento. A prevenção, cirurgia e a rádio/quimioterapia são as principais abordagens usadas no combate à doença. É importante encontrar novos tratamentos mais eficazes, específicos e melhor suportados pelos pacientes.

Novas estratégias para serem usadas contra o CCR surgiram aquando da descoberta de que algumas estirpes do género *Propionibacterium* poderiam fornecer proteção contra o CCR. Essas bactérias, que vivem no intestino do Homem, produzem ácidos gordos de cadeia curta, como o acetato, o butirato e o propionato, que são capazes de induzir a apoptose específica das células do CCR. Os mecanismos que levam a essas ocorrências não estão bem compreendidos. A ceramida é uma molécula de sinalização importante e vital em processos celulares, e poderá desempenhar um papel na regulação da apoptose em células de CCR. Investigação prévia do nosso laboratório, mostrou que o acetato induz apoptose em linhas de CCR, bem como a permeabilização da membrana lisossomal (PML) com libertação de catepsina D. O facto de se saber que a ceramida promove a PML e activa a catepsina D, levou-nos a colocar a hipótese de que a ceramida poderia ser gerada em resposta ao acetato e poderia mediar a apoptose induzida por esse estímulo letal, em células do CCR.

O principal objectivo do projeto foi avaliar o envolvimento da ceramida na apoptose induzida pelo acetato, em células do CCR (HCT-15 e RKO). Para atingir essa meta, as células foram tratadas com acetato e um ou mais inibidores das vias de síntese da ceramida (GW4869, fumonisin B_1 e myriocin) e a viabilidade celular foi analisada recorrendo a ensaios de MTT.

A inibição das três principais vias de produção de ceramida (*de novo*, selvagem e das esfingomielinases) não alterou significativamente a viabilidade celular em ambas as linhas celulares. De facto, o esperado aumento na viabilidade celular não ocorreu quando as células foram tratadas com acetato e com um ou mais inibidores das vias de síntese da ceramida. Mais estudos são necessários para confirmar os resultados obtidos, nomeadamente a determinação dos níveis celulares da ceramida e a actividade de enzimas envolvidas na síntese da mesma em células tratadas com acetato e um ou mais inibidores das vias de síntese da ceramida.

Resumindo, os resultados obtidos até agora sugerem que a morte celular induzida pelo acetato em células HCT-15 e RKO de CCR parece não ser mediada pela ceramida.

Contents:

List of Abbreviations:

µL: Microlitre

µM: Micromolar

Akt: Protein kinase B (synonym of PKB)

ANT: Adenine nucleotide translocator

AP1: Activator protein 1

APC: Adenomatous polyposis coli

APO-1: Apoptosis antigen 1

aSMase: Acid sphingomyelinase

ATCC: American type culture collection

BID: BH3 interacting-domain death agonist

BMI: Body mass index

BRAF: v-Raf murine sarcoma viral oncogene homolog B1

BrdU: Bromodeoxyuridine (5-bromo-2'-deoxyuridine)

C1PP: Ceramide-1-phosphatase

CAPK: Ceramide activated protein kinases

CAPP: Ceramide activated phosphatase

CD95: Cluster of differentiation 95 (synonym of APO-1)

CDase: Ceramidase

CerS: Ceramide synthase

CIN: Chromosomal instability

CK: Ceramide kinase

CO_2: Carbon dioxide

CRC: Colorectal carcinoma

CRS: Cerebrosidase

DAG: Diacylglycerol

DES: Dihydroceramide desaturase

DISC: Death inducing signalling complex

DMH: 1,2-dimethylhydrazine

DMSO: Dimethyl sulfoxide

ED_{50}: Half maximal effective concentration

ER: Endoplasmic reticulum

ERK: Extracellular signal-regulated kinase

FACS: Fluorescence-activated cell sorting

FAP: Familial adenomatous polyposis

FB_1: Fumonisin B_1

GCS: Glucosylceramide synthase

GW: GW4869

h: Hours

H_2O_2: Hydrogen peroxide

HMA: Human-microbiota associated

HNPCC: Hereditary nonpolyposis colorectal cancer

IC_{50}: Half maximal inhibitory concentration

IGF2R: Insuline-like growth factor 2 receptor

IPATIMUP: Institute of Molecular Pathology and Immunology of the University of Porto

JNK1: Mitogen-activated protein kinase 8 (MAPK8 synonym)

KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

LDL: Low-density lipoprotein

LMP: Lysosomal membrane permeabilization

M: Molar

MAMs: Mitochondria-associated membranes

microRNA: Micro ribonucleic acid

min: Minutes

mL: Millilitre

mM: Milimolar

MMP: Mitochondrial membrane permeabilization

MMR: Mismatch repair

MOMP: Mitochondria outer membrane permeabilization

MSI: Microsatellite instability

mTOR: Mammalian target of rapamycin

MTT: Methyl-thiazolyl-tetrazolium (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

Myr: myriocin

NF-Kβ: Nuclear factor Kβ

nM: Nanomolar

nSMase: Neutral sphingomyelinase

O_2: Oxygen

p53: protein 53 (or tumour protein 53)

PBS: Phosphate buffered saline (solution)

PC: Phosphatidylcholine

PI: Propidium iodide

PKB: Protein kinase B

PKC: Protein kinase C

PP1: Protein phosphatase 1

PP2A: Protein phosphatase 2A

PTPC: Permeability transition pore complex

Rb: Retinoblastoma (protein)

ROS: Reactive oxygen species

***S.cerevisiae*:** *Saccharomyces cerevisiae*

S1P: Sphingosine-1-phosphate

S1PP: S1P phosphatase

SCFA: Short-chain fatty acid

SD: Standard deviation

SEM: Standard error of the mean

SK or SphK: Sphingosine kinase

SMase: Sphingomyelinase

SMS: Sphingomyelin synthase

Sph: Sphingosine

SPT: Serine palmitoyltransferase

SRB: Sulforhodamine B

TGFβ: Transforming growth factor-β

TGFβR2: TGFβ receptor 2

TLC: Thin layer chromatography

TNF: Tumour necrosis factor

TNF-α: Tumour necrosis factor-α

TRAIL: TNF-α-related apoptosis-inducing ligand

***t*-test:** Student's *t* test

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labelling

UV: Ultraviolet

VDAC: Voltage-dependent anion channel

vMIA: Viral mitochondrial inhibitor of apoptosis

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1

General Introduction

1.1 Cancer: incidence, genetics and hallmarks

Nowadays, in the modern and developed countries and increasingly in developing nations, cancer is a disease with great incidence and affecting a growing number of people.

In 2008, 12.7 million of new cases of cancer and 7.6 million cancer deaths were estimated, with 56% and 63% of new cases of cancer and cancer deaths, respectively, occurring in the less developed regions of the world (Ferlay, Shin *et al.*, 2010). A new tendency has been noticed, while mortality rates are decreasing in many western countries, like the Unites States of America, this rate is growing in less developed and economically transitioning nations, due mostly to the adoption of unhealthy habits more known to western lifestyles (Jemal, Center *et al.*, 2010). More particularly, in Europe, 3.2 million of new cases of cancer and 1.7 millions of resulting deaths were estimated, being colorectal cancers the most common form of the disease found (13.6% of the total) and also the second most common cause of death (12.3% of the total) (Ferlay, Parkin *et al.*, 2010).

Cancer is a complex disease with multiple causes. Nevertheless it could be defined as an atypical growth of cells, caused by several changes in gene expression leading to a disequilibrium between cell proliferation and cell death and, in some advanced cases, evolving into a population of cells capable of tissue invasion and metastization, far from the original location, resulting in an elevated morbidity and, if not treated, death of the host (Ruddon, 2007).

Several events are required to transform a normal cell into a malignant one and such process is called carcinogenesis. As a multi-step disease, cancer is caused by alterations that generally occur in somatic cells (although mutations in germ-line cells can also prompt a person to inherited cancer) affecting oncogenes, tumour suppressor and microRNA genes (Croce, 2008).

Some traits seemed to be shared by most forms of tumours. In fact, these acquired capabilities are the main consecutive alterations that enable carcinogenesis. There are six principal hallmarks that dictate cancer growth: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis - Figure 1.1 (Hanahan and Weinberg, 2000). More recently, two other characteristics, involved in the pathogenesis of some and maybe all cancers, were added to the previous list, as emerging hallmarks: reprogramming of energy metabolism and evading immune destruction. Additionally, tumour-promoting inflammation and genomic instability and mutation are consequential properties of tumours that facilitate the acquisition of core and emerging hallmarks - Figure 1.1 (Hanahan and Weinberg, 2011).

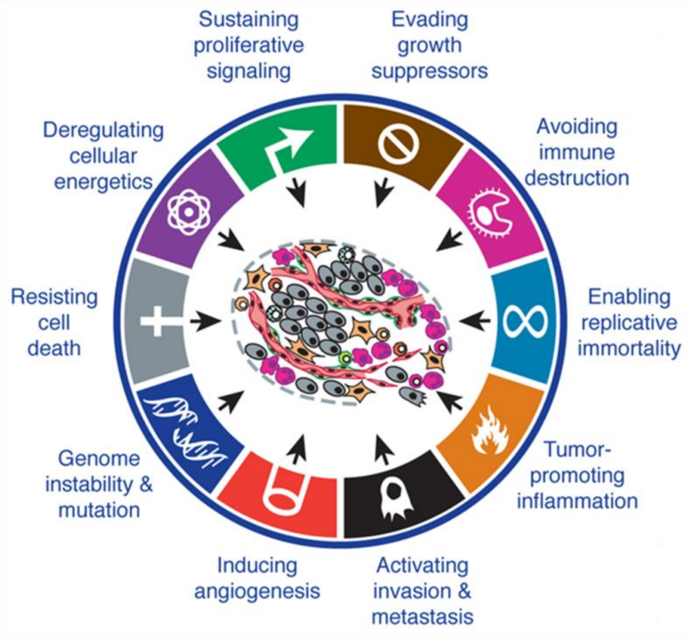


Figure 1.1 – The hallmarks of cancer, emerging hallmarks and enabling characteristics. Adapted from (Hanahan and Weinberg, 2011).

1.2 Colorectal carcinoma

Among all the different forms of cancer, colorectal carcinoma (CRC) has a great incidence and mortality in world population. According to the GLOBOCAN studies, in 2008, (Ferlay, Shin *et al.*, 2010), CRC was the third most common type of cancer and the fourth cause of death, in both sexes, worldwide, with a major prevalence (60%) in developed countries.

There are two principal forms of colorectal cancer: the sporadic form and the inherited forms, which include the familial adenomatous polyposis (FAP) and the hereditary nonpolyposis colorectal cancer (HNPCC) (Potter, 1999).

The sporadic form is the most frequent form of colorectal cancer (70% of the cases), beginning from somatic mutations and evolving into a tumour. Inherited susceptibility to colorectal cancer may underlie 30% of all diagnosed types: 5% with an evident hereditary base predisposing to the disease and the remaining percentage only showing an elevated propensity of family members to be affected by the disease, without in fact presenting recognizable hereditary syndromes (Rustgi, 2007; Tops, Wijnen *et al.*, 2009). FAP is an autosomal dominant inherited disease, characterized by the emergence of hundreds to thousands of adenomas or adenomatous polyps, during the second and third decade of patients' lives. Even though these tumours are usually benign and individually not life threatening, some will eventually progress and origin malignant forms of cancer (Kinzler and Vogelstein, 1996). HNPCC (or also called Lynch syndrome), is another inherited autosomal dominant syndrome, that exhibits a much less well characteristic phenotype than FAP and is often confused with sporadic polyposis (Potter, 1999). HNPCC involves a predisposition not only to colorectal cancer, but also to other seven types of cancer, among them: the small bowel, endometrium, ovaries, stomach, brain, hepatobiliary epithelium and uroepithelial epithelium (Lynch, Guirgis *et al.*, 1977). The FAP and HNPCC conditions account to 5% or less of total CRC cancers: less than 1% for FAP and, approximately, 5% for HNPCC (de la Chapelle, 2004).

1.2.1 Colorectal carcinogenesis

Many genetic and structural alterations are necessary to the development of colorectal carcinoma. In 1990, Fearon and Vogelstein proposed a genetic model for colorectal tumourigenesis. That model evokes a sequential multistep changes, affecting several types of genes, represented in Figure 1.2, and that would consequently lead to progressive morphologic shifts of the colon and rectal apparatus (Fearon and Vogelstein, 1990).

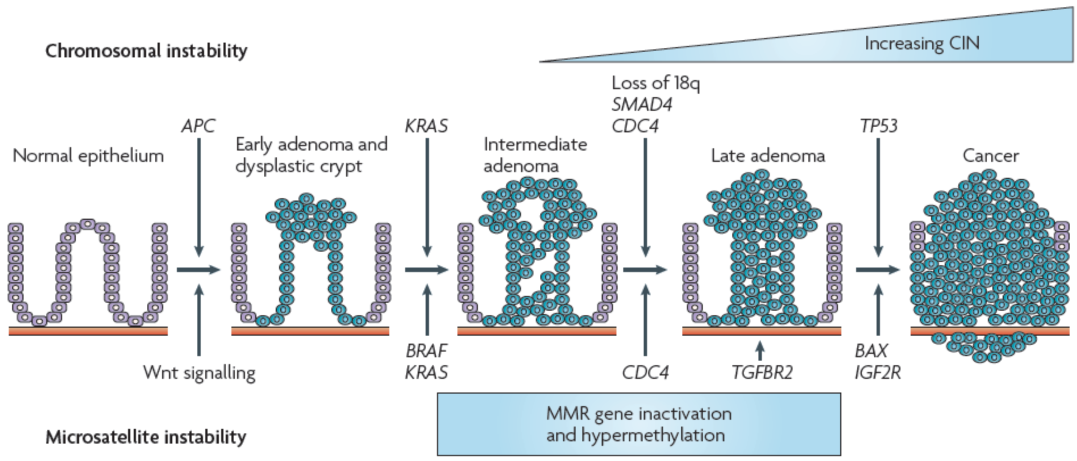


Figure 1.2 – Adenoma-carcinoma sequential model for chromosomal instability, in colorectal cancer. Adapted from (Walther, Johnstone *et al.*, 2009). *APC*: adenomatous polyposis coli; TGFβ: transforming growth factor – β; MSI: microsatellite instability; CIN: chromosomal instability; MMR: mismatch repair; TGFβR2: transforming growth factor – β receptor 2; IGF2R: insulin-like growth factor 2 receptor.

To guarantee the CRC evolution, at least, four sequential genetic alterations or "hits" in specific oncogenes and tumour-suppressor genes need to occur. *KRAS*, an oncogene, and *APC*, *SMAD4* and *TP53*, three tumour-suppressor genes are the main targets of these changes (Fodde, Smits *et al.*, 2001).

The *APC* gene has a key role in CRC. Mutations of the germ-line in the *APC* gene are responsible for the FAP and are also found in non-hereditary colorectal cancers (Tanaka, 2009). The *APC* gene encodes a protein that has a preponderant role in Wnt signalling pathway, given that *APC* regulates cell proliferation. The mechanism by which this regulation is made depends on APC binding and degrading of β-catenin to promote cell proliferation (Ilyas, 2005). If *APC* is mutated this cannot occur and β-catenin is translocated to the nucleus, where it binds to a transcription factor (T-cell factor/lymphocyte enhancer factor). This will activate *c-myc*, *cyclin D1* and *c-junc* genes and induce cell proliferation (Tanaka, 2009).

Other important functions have been attributed to APC such as the control of intracellular adhesion, that might be achieved by regulating the stability and subcellular localization of β-catenin (a constituent of adherens junctions), being the link between α-catenin and E-cadherin, that binds actin and its associated proteins (Ben-Ze'ev and Geiger, 1998). Furthermore, the direct association between APC with the microtubule cytoskeleton was also demonstrated (Munemitsu, Souza *et al.*, 1994; Smith, Levy *et al.*, 1994).

KRAS gene is also an important piece in the carcinogenesis process of CRC. It encodes a protein involved in G protein-mediated transduction. KRAS protein has a GTPase activity, which transmit signal across the cellular plasma membrane through the activation of the RAS-RAF-MEK-ERK-MAPK signalling cascade (Roberts and Der, 2007). The most widespread types of *KRAS* mutation, in colorectal cancers, are the substitution of glycine to aspartate on codon 12 (p.G12D), glycine to valine on codon 12 (pG12V) and glycine to aspartate on codon 13 (p.G13D) (Neumann, Zeindl-Eberhart *et al.*, 2009). Mutation of *KRAS* is responsible for unregulated and increased cell proliferation and malignant transformation (Bos, 1989).

BRAF mutations have been identified in several types of human cancers, including colorectal tumours. The frequency of this mutation associated to CRC varies between 1% and 20%, and *BRAF* mutation is specially connected to tumours with a dysfunction in mismatch repair (MMR) activity (Wellbrock, Karasarides *et al.*, 2004). A transversion (T into A) is the most common *BRAF* mutation (90% of *BRAF* mutant tumours), originating a valine to a glutamic acid alteration - V600E (Davies, Bignell *et al.*, 2002). In consequence, there is a major increase in *in vitro* kinase activity and the stimulation of cell proliferation and transformation (Wan, Garnett *et al.*, 2004). Recently, it has been demonstrated that the inhibition of *BRAF* in CRC cell lines interfered with the proliferation and apoptosis mechanisms. In *BRAF*^{V600E} microsatellite unstable (MSI) cell lines, the suppression of BRAF, using the RNA interference technique, significantly induced apoptosis and inhibited proliferation. In contrast, no significant differences were observed in proliferation and apoptosis in cell lines harbouring a *KRAS*^{G13D} mutation, when submitted to BRAF inhibition (Preto, Figueiredo *et al.*, 2008). Besides, the study from Preto and collaborators also found that, in *BRAF*^{V600E} cell lines under BRAF inhibition, the levels of proliferation associated molecule p27^{Kip1} are increased and the levels of the anti-apoptotic protein Bcl-2 are decreased.

The mutation of the *TP53* gene is preponderant not only in CRC cases, but also in the majority of known cancers. It is thought to be a relatively late event in the carcinogenesis process of CRC and determinant in the progression from adenoma to a malignant tumour. This gene is mutated in up to 70% of colorectal cancers (Baker, Preisinger *et al.*, 1990). In 1979, *TP53* tumour suppressor gene was the first one to be identified. It encodes a transcription factor - p53 protein - implicated in cell cycle control. The p53 protein specifically binds to a recognition sequence in a variety of genes, including p21, Bax, and Bcl-2, when the DNA is damaged or the cell is under stress, if not the p53 network is usually not active (Vogelstein, Lane *et al.*, 2000). p53 has a protective role toward cells, as well as a destructive one. Different stimuli, such as basal low-levels of p53, stress, DNA damage and oncogenic signals can all elicit the p53 system, resulting in different cell responses such as transient cell cycle arrest, autophagy, repair, apoptosis, senescence and necrosis. It is clear, that a shift in p53 output, from a death signalling to a repair one, can promote survival of mutated and damaged cells and increase the probability of tumour cell survival (Junttila and Evan, 2009).

The *APC*, *KRAS*, *BRAF* and *TP53* are thought to be the major mutations found in CRC, but the carcinogenesis process is a complex set of events and all the interveners are not known. Additionally, we must be aware of the cross-talk between pathways that augment the tumourigenesis complexity. The more profound is our knowledge about CRC mutations, the bigger is the probability to find new strategies and new approaches to cancer therapy.

1.2.2 Colorectal carcinoma: risk factors, prevention and therapy

The survival rates of CRC have significantly improved in the past years. The acquired knowledge about the disease, the prevention, the early detection and the development of treatments are fundamental reasons that sustain this increase.

A crucial part in the diminution of the CRC incidence is the identification of potential dangerous factors that contribute to the malady apparition. Risk factors behind CRC development can be divided in two categories: non-modifiable and modifiable risk factors. Non-modifiable risks factor are age and predispositions factors (incidental polyps, inherited disorders, inflammatory bowel disease, some of these already discussed in the last section). The major part of CRC cases occur in older individuals (age above 50 years), principally because the carcinogenesis process requires several mutations that gather over time. It is known that it would take up to ten years for cells to undergo significant mutations and become malignant (Scholefield, Ritchie *et al.*, 2005). Interestingly ethnicity is also considered a non-modifiable risk factor for CRC. For example, the Ashkemazi Jews are a highly predisposed group in terms of the disease development, due to the I1307K *APC* mutation that runs among (6-7%) of the population (Locker, Kaul *et al.*, 2006).

Worldwide patterns and variations of CRC incidence are also consequence of modifiable risks. Obesity is an undeniable risk factor for CRC. Some studies showed that the body mass index (BMI) is connected to the incidence of CRC, and that risk suffers an elevation of 25% and 50% in overweight and obese men, respectively (Moghaddam, Woodward *et al.*, 2007). The link between the BMI and colorectal cancers seems to be weaker in women (Murphy, Calle *et al.*, 2000). If obesity is a risk factor, certainly the lack of physical activity is also a factor to be considered, as they are tightly linked. The presence of central adiposity and the absence of regular physical activity is associated with poorer survival rates, after CRC diagnosis (Haydon, MacInnis *et al.*, 2006).

Diet has proven to be a decisive feature to either prevent or be, at least, in part responsible for the development of colorectal cancers. Currently, it is believed that a diet constituted of red meat with low portions of vegetables and fruits are likely to be increasing the risk of cancer (van Duijnhoven, Bueno-De-Mesquita *et al.*, 2009).

The two last modifiable factor risks important to be referred are alcohol consumption and smoking. The uncontrolled alcohol consumption on a regular basis, as well as the habit of smoking, are thought to be associated with the increase in CRC incidence. An increased risk of 16% and 41% is observed in individuals that drink 30-45 g/day and ≥45 g/day, respectively (Cho, Smith-Warner *et al.*, 2004). Concerning the smoking, it very well known that several carcinogenic compounds are found in tobacco, being an important factor in CRC, despite being far more preponderant in other cancer types (Liang, Chen *et al.*, 2009).

Having in mind all the risks factors, prevention seems the best way to avoid and decrease the incidence of colorectal cancers. Several research works revealed that diet, as discussed above, could be important to prevent CRC and other form of tumours. Phytochemicals, included in the chemopreventive agent's category, are found in vegetables and fruits and demonstrated to have a preventive effect on proliferation, to induce apoptosis and to inhibit growth factor signalling pathways. In addition, phytochemicals play a role in reversing chemoresistance and radioresistance, among other positive effects (Dorai and Aggarwal, 2004). A spice, curcumin, common in India has proven to be relevant in CRC prevention by promoting apoptosis, cell cycle arrest and participate in other essential pathways too (Johnson and Mukhtar, 2007). Resveratrol (grapes), capsaicin (chilli peper) and gingerol (ginger) are all natural compounds able to stop tumour cell proliferation and malignant transformation by targeting and suppressing the overexpression of NF-κB (nuclear factor κB) and AP1 (activator protein 1) transcription factors, respectively (Surh, 2003). In addition, the dietary phytochemicals quercetin, luteolin and ursolic acid demonstrated to have anti-proliferative and pro-apoptotic effects on CRC derived cell lines (HCT-15 and CO115) and seemed to act on KRAS and PI3K. Quercetin and luteolin decreased ERK (extracellular-signal-regulated kinase) phosphorylation, in HCT-15 cells, while the three compounds led to decreased Akt phosphorylation on CO115 cells (Xavier, Lima *et al.*, 2009).

A set of products called probiotics and prebiotics are thought to be relevant in the prevention of colorectal carcinoma, for example, the consumption of milk and its derivatives, products containing lactobacilli or bifidobacteria, seems to diminish the CRC incidence (Wollowski, Rechkemmer *et al.*, 2001). Among the effects of pre and probiotics, that appear to reduce the risk of CRC, we can count the changes in the pH conditions in the colon, the lower production of probable carcinogenic product by gut microflora, the modulation of the immune response, the production of anticancer compounds, inter alia (Lim, Ferguson *et al.*, 2005; Fotiadis, Stoidis *et al.*, 2008).

Chemopreventive agents are also able to improve the efficiency of common cancer therapy i.e. the combined use of preventive agents and chemotherapy or radiotherapy (Sarkar and Li, 2006). Further investigation is needed to understand the mechanisms of synergy between chemopreventive agents and usual therapy, to take benefit of it.

1.3. Relation between intestinal flora and colorectal carcinoma

Human intestines are colonized by a large and diverse population of microorganisms. Bacteria are the predominant type and are indispensable for our survival and the healthy functioning of our organism. Gut microflora is responsible for multiple tasks as, for instance, saving nutrients and energy acquired from food, controlling the epithelial cell proliferation and differentiation, balancing the immune system homeostasis and protecting, in certain cases, against possible pathogens (Guarner and Malagelada, 2003).

The intestinal flora influence on colorectal carcinogenesis has become increasingly evident. Bacteria have been associated to CRC by production of toxic and genotoxic metabolites that affect intracellular signal transduction (Fasano, 1999). N-Nitroso compounds and bile acids are examples of such metabolites (Rowland, 2009). Other studies show that the metabolic activity of gut microbiota produces toxic products, such as reactive oxygen species (ROS), which lead to DNA damage and chronic inflammation contributing to tumourigenesis. Increasing findings demonstrated that the same agents are implicated in changes in host glycosylation, altering the cellular and sub-cellular distribution of glycans, and those alterations are linked to the neoplastic process (Hope, Hold *et al.*, 2005).

On the other hand, short chain fatty acids (SCFAs) – acetate, propionate and butyrate – seem to have a protective effect against colorectal carcinoma (Scheppach, 1994), as it will be addressed in the next sections.

1.3.1 Bacterial production of acetate in human gut

A substantial part of carbohydrates consumed in human daily routine are fermented by several microorganisms in the colon. This is a result of the ingestion of cellulose (plant cell wall polysaccharides), hemicelluloses and pectins, vulgarly known as dietary fibre (Cummings, 1981).

Bacteria present in human gut are capable of fermentation, not only of carbohydrates (polysaccharide and oligosaccharide) but also of proteins, peptides and glycoproteins precursors in the colon, resulting into pyruvate formation and its conversion into a series of intermediary and end products such as butyrate (butyric acid), acetate (acetic acid), propionate (propionic acid), carbon dioxide, methane, hydrogen and water (Miller and Wolin, 1979; Cummings, 1981).

Between SCFAs products, acetate is the most produced (Cummings and Macfarlane, 1991). There are a vast set of bacteria species involved in acetate formation; among them are the following ones: *Anaerotruncus colihominis*, *Bacteroides goldsteinii*, *Bifidobacterium*, *Cetobacterium somerae*, *Clostridium asparagiforme*, *Clostridium hathewayi*, *Dorea longicatena*, *Eubacterium*, *Lactbacillus*, *Streptococcus*, *Ruminococcus luti*, and *Victivallis vadensis* (Miller and Wolin, 1979; Duncan, Louis *et al.*, 2007).

Short chain fatty acids are absorbed in various zones of the colon and later metabolized at three specific sites in the organism: the ceco-colonic epithelium, the liver and muscles (Hijova and Chmelarova, 2007). Butyrate is used as a substrate to maintain the energy of producing pathways by cells belonging to the ceco-colonic epithelium. In the liver, SCFAs (residual butyrate and propionate) are metabolized and used in gluconeogenesis, also a great part of acetate (50% to 70%) is taken up by this organ. Additionally, a residual amount of acetate is utilized in muscle cells to generate energy from its oxidation. In general, SCFAs are important nutrients for the colonic epithelium cells. They have a role not only in the regulation of gene expression, cell proliferation and differentiation but also as modulators of colon ion transport, cell volume and intracellular pH (Cook and Sellin, 1998). As these compounds are rapidly absorbed in the colon, they are connected to bicarbonate excretion (Binder, Rajendran *et al.*, 2005) and improve sodium absorption (Trinidad, Wolever *et al.*, 1996).

Focusing in acetate metabolism, it is known that it is absorbed in the colon and transported to the liver. Once it enters the systemic circulation, acetate can be a part of lipogenesis that occurs in mammary glands (Hijova and Chmelarova, 2007). Acetate is the most abundant SCFA in the blood and it is the primary substrate for the synthesis of cholesterol, as well as it might be absorbed and used in peripheral tissues (Pomare, Branch *et al.*, 1985).

1.3.2 The role of acetate and other SCFAs in colorectal carcinoma cell death

As active compounds in the organism, SCFAs could have a beneficial effect on health especially in the fight against CRC. In fact, research on this area is evolving and every day a little more is known on the subject.

Sources of SCFAs that come from alimentation are nearly entirely dairy products. Once in the organism, they have numerous effects on colonic epithelial cells at different moments: growth, development, transformation and cell death (Cook and Sellin, 1998). Such interactions could explain the preventive effect of SCFAs on colorectal carcinoma (Figure 1.3).

Studies support the notion that production of endogenous SCFAs might diminish the risk of cancer in colonocytes through processes such as apoptosis (Heerdt, Houston *et al.*, 1994) and regulation of histone acetylation (Hinnebusch, Meng *et al.*, 2002). Some experiments realized with HT-29 cells, confirmed that butyrate and propionate are able to enhance histone acetylation and might contribute to biological effects connected to cancer prevention or progression (Kiefer, Beyer-Sehlmeyer *et al.*, 2006).

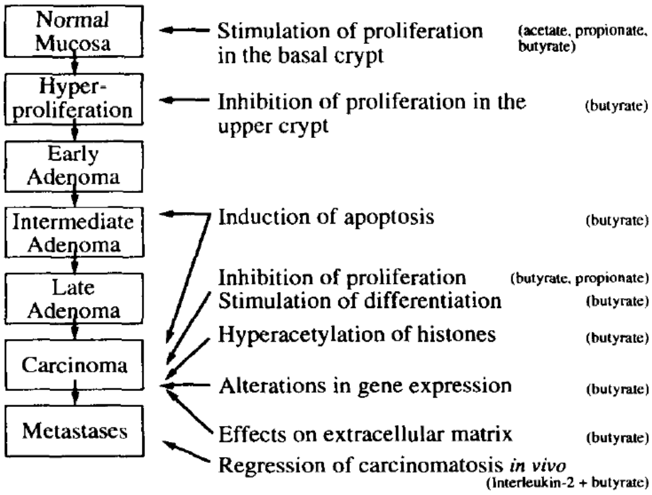


Figure 1.3 – Effects of short-chain fatty acids (SCFAs), on colonic epithelial cells, at different phases of the adenoma-carcinoma sequence. Adapted from (Scheppach, Bartram *et al.*, 1995).

On the other hand, programmed cell death is a fundamental process to keep the balance between cell generation and cell death. Interruption or defects on this process would alter this balance and favour tumour progression. All three major SCFAs, acetate, butyrate and propionate, have all proven to induce apoptosis in several adenoma and carcinoma cell lines, but butyrate seemed to be the most efficient and the most studied (McBain, Eastman *et al.*, 1997; Cook and Sellin, 1998). Some interesting studies have been made showing, for instance, that propionibacteria are able to induce apoptosis, *in vitro*, in two colorectal carcinoma cell lines (HT-29 and Caco 2) by means of acetate and propionate produced by fermentation (Jan, Belzacq *et al.*, 2002). Two species of bacteria were object of the study *P. acidipropionici* and *P. freudenreichii*. The effects of propionate and acetate were tested alone or in combination, with a half maximal effective concentration (ED_{50}) of 11 mM and 20 mM respectively, in HT-29 cells. The SCFAs-induced apoptosis was confirmed by the observation of ROS generation, caspase-3 processing and nuclear chromatin condensation. In addition, the oncoprotein Bcl-2 (known to prevent apoptosis *via* mitochondria) and the viral mitochondrial inhibitor of apoptosis (vMIA), which interacts with the adenine nucleotide translocator (ANT), both inhibited cell death induced by SCFAs, suggesting that mitochondria is decisively involved in this cell death process (Jan, Belzacq *et al.*, 2002). Others studies also denote the induction of apoptosis of CRC cells by SCFAs (acetate and propionate) involving the mitochondria (Heerdt, Houston *et al.*, 1997; Jan, Belzacq *et al.*, 2002). It is evident that this process may be decisive in the attempt of finding a manner to prevent or treat colorectal cancers.

Another investigation reinforces this finding demonstrating a main impact of a shift in extracellular pH, on the mode of propionibacteria SCFA-induced cell death of HT-29 cells, leading to SCFA-induced cell cycle arrest and apoptosis (Lan, Lagadic-Gossmann *et al.*, 2007) and even leading to apoptosis *in vivo* (Lan, Bruneau *et al.*, 2008). In 2007, Lan and collaborators demonstrated that *P. freudenreichii* SCFAs led to apoptosis and induced cell cycle arrest in G2/M phase prior to programmed cell death at pH 7.5. This pH also caused all typical morphological changes known in apoptosis (membrane blebbing, chromatin condensation and fragmentation, phosphatidylserine exposure and formation of apoptotic bodies). Otherwise at a pH of 5.5, the necrotic process was observed, in HT-29 cells, manifested by rapid swelling and disruption of internal organelles and plasma membrane lyses without chromatin fragmentation (Lan, Lagadic-Gossmann *et al.*, 2007).

In 2008, Lan and co-workers also noticed that *Propionibacterium freudenreichii* could be able of not only killing cells *in vitro*, but also *in vivo*, with a remarkable selectivity. Apoptosis and proliferation of colonic epithelial cells were analysed in human microbiota-associated (HMA) rats, fed with *P. freudenreichii* TL133 strain on a daily routine, 48h hours after the induction of carcinogenesis with 1,2-dimethylhydrazine (DMH). The strain survived in the gastrointestinal tract of rats and demonstrated to induce apoptosis only in DMH-damaged cells, revealing the specificity of these bacteria protective role (Lan, Bruneau *et al.*, 2008).

Recent results, obtained by our laboratory, showed that SCFA and more particularly acetate is able to in induce apoptosis and to inhibit proliferation in HCT-15 and RKO CRC derived cell lines. This SCFA is also responsible for triggering lysosomal membrane permeabilization (LMP) and cathepsin D release to the cytosol (Marques, Oliveira *et al.*, 2012 ; submitted).

All the combined results showed above contribute to the idea that probiotics, such as propionibacteria and their respective metabolic products, could be used as strong agents for colorectal carcinoma prevention and perhaps therapy.

1.4 Apoptosis: the programmed cell death

Apoptosis is a cellular process of programmed cell death and is deeply important in development, immune response and tissue homeostasis. More and more studies link this process to the successful triggering of cancer cells death, namely CRC cells, when those seem "immortal".

This type of death is characterized by a set of morphological changes beginning with cell shrinkage and chromatin condensation. Cell blebbing occurs and the "budding" process takes place, in other words, cell fragments are separated into apoptotic bodies that consists of cytoplasm (enclosed within an intact plasma membrane), containing tightly packed organelles with or without nuclear fragments (Elmore, 2007). These changes are caused by several cysteine proteases, known as caspases, that participate in two (intrinsic and extrinsic) pathways described for apoptosis (Figure 1.4).

The intrinsic and extrinsic pathways of apoptosis differ in some points. The first pathway is activated by many different types of cellular stress, including some chemotherapeutic compounds, UV and γ-irradiation, reactive oxygen species (ROS), radicals generated as by-product of normal cell metabolism, oncogene activation and DNA damage. Basically, the death stimuli origin comes from within the cell (Yang, Sales *et al.*, 2009). Mitochondria has a central role in this pathway, as the apoptotic stimuli converge in the mitochondrial membrane permeabilization (MMP), releasing some pro-apoptotic proteins into the cytoplasm (such as cytochrome *c*), thereby triggering the activation of a cascade of caspases (Kroemer and Reed, 2000; Riedl and Shi, 2004).

On the other hand, the extrinsic pathway is activated by the so-called "death ligands", for example, tumour necrosis factor-α (TNF-α) and TNF-α-related apoptosis-inducing ligand (TRAIL) (Yang, Sales *et al.*, 2009). When they bind to their "death receptor", a protein complex, called the death inducing signalling complex (DISC), is formed and the activation of the caspase cascade occurs (Riedl and Shi, 2004). In some cases, activation of caspase-8 directly leads to caspase cascade launch while, in other situations, caspase-8 modulates apoptosis through the cleavage of BH3 interacting-domain death agonist (BID), which in turn allows the MMP and consequent programmed cell death (Li, Zhu *et al.*, 1998).

The programmed cell death is object of a rigorous control. This regulation include proteins of the Bcl-2 family, that contains both agonists (Bax, Bak, Bad, Bcl-X_s) and antagonists

(Bcl-2, Bcl-X_L) of apoptosis (Ligr, Madeo *et al.*, 1998). Those proteins also exercise control over MMP enhancing or inhibiting it (Kroemer, 2003).

Besides mitochondria, other organelles and cellular compartments have been associated to the regulation of apoptosis: the endoplasmic reticulum (ER) and the lysosome.

The stress upon the ER and its dysfunction (due to the accumulation and aggregation of unfolded proteins, for example) can result in cellular death, through apoptosis, if the stress is prolonged and the response fails restoring the normal ER function (Szegezdi, Logue *et al.*, 2006).

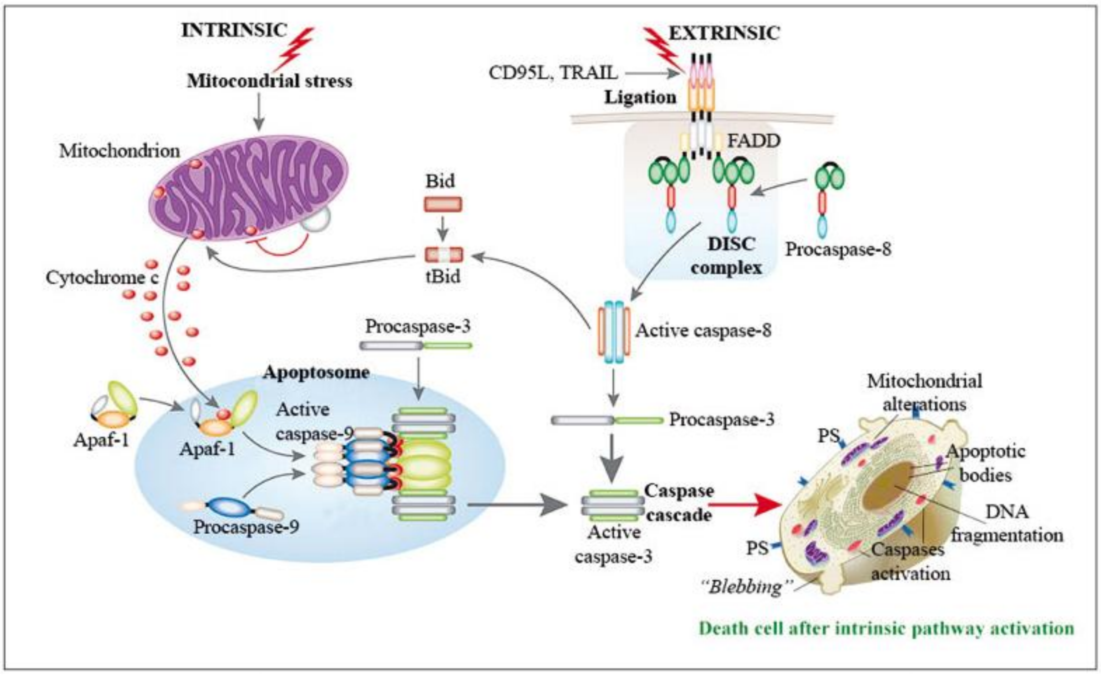


Figure 1.4 – Representation of the apoptotic intrinsic and extrinsic pathways. Adapted from (Calvino-Fernández and Parra-Cid, 2010).

The lysosome is also involved in programmed cell death. In response of certain stimuli, including ROS, TNF receptor ligation, p53 activation and lipid second messenger sphingosine, lysosome partial membrane permeabilization has been shown to initiate an apoptosis response (Jäättelä, Candé *et al.*, 2004; Boya and Kroemer, 2008). After stimulation, lysosomal membrane permeabilization (LMP) occurs through caspase-dependent mechanisms and releases cathepsins and other hydrolases in the cytosol (Kroemer, Galluzzi *et al.*, 2007). It is thought that cathepsins are able to cleave Bid, leading to Bax activation and consequent release of apoptotic factors from mitochondria, initiating the programmed death of the cell (Cirman, Oresic *et al.*, 2004).

1.4.1 Apoptosis in yeast and its relation to mammalian and colorectal carcinoma cells

Over the years, the apoptosis process has been studied using a not so complex model such as the mammalian one: *Saccharomyces cerevisiae* has been largely used as a eukaryotic model for the understanding of programmed cell death.

In *S.cerevisiae*, the apoptotic phenotype was found to be triggered by acetic acid (an end product of the alcoholic fermentation realized by this type of yeast), involving mitochondria and the release of cytochrome *c* in the process (Ludovico, Sousa *et al.*, 2001; Ludovico, Rodrigues *et al.*, 2002). It was also demonstrated that acetic acid had a stimulating effect on the mitochondrial outer membrane permeabilization (MOMP), requiring ADP/ATP carrier (AAC) proteins, which are orthologues of the adenine nucleotide translocator (ANT) of mammalian cells (Pereira, Camougrand *et al.*, 2007). Moreover, cell death triggered by acetic acid in yeast was found to be responsible for the Pep4p (orthologue of mammalian cathepsin D) translocation from the vacuole to the cytosol (Pereira, Chaves *et al.*, 2010).

The mechanisms described above, also happen in mammalian cells. It is believed that MOMP event is due, at least in part, to the formation of the permeability transition pore complex (PTPC), composed by several proteins, in which ANT and the voltage-dependent anion channel (VDAC) are included. The PTPC formation occurs between the two mitochondrial membranes, ANT and VDAC being located in the inner and the outer membrane, respectively (Halestrap, McStay *et al.*, 2002). And with the opening of PTPC, proteins (such as cytochrome *c*) are released and apoptosis is engaged (Henry-Mowatt, Dive *et al.*, 2004).

The fact that similar events occurs both in yeast and mammalian cells is interesting, but is acetic acid/acetate capable of triggering such events in mammalian cells to? As mentioned in a previous section (1.3.2), evidences point to a certain kind of bacteria, more precisely propionibacteria, that are able to survive in the human intestines and kill colorectal carcinoma cells. These microorganisms, through their metabolism, produce metabolites, SCFAs (acetate, butyrate and propionate) that seem to be responsible for the death of CRC cells, by triggering apoptosis.

In results obtained by our group, it was found that acetate lead to CRC apoptosis and that LMP and release of cathepsin D (to the cytosol) occurred in CRC cells undergoing apoptosis (Marques, 2010 ; Master thesis). The inhibition of cathepsin D with pepstatin A proved to lead to the increase in acetate-induced apoptosis in the HCT-15 and RKO CRC derived cell lines

(Marques, Oliveira *et al.*, 2012 ; submitted). In a yeast model, in accordance with the mammalian studies, deletion of the Pep4p confers higher susceptibility to acetic acid, while the overexpression of this molecule confers higher resistance to yeast cells (Pereira, Chaves *et al.*, 2010). Thus, the role of cathepsin D may be protective over the process of acetate-induced apoptosis. Moreover, the overexpression of cathepsins have been linked to cancer, poor prognosis and higher risk of recurrence (Palermo and Joyce, 2008), although studies have been showing the emerging role of these proteases as tumour suppressor (López-Otín and Matrisian, 2007).

Every day, more and more is known about the mechanisms and routes of signalizations used by SCFAs to induce programmed cell death, but more studies are needed in order to unveil such processes and maybe provide a new strategy of CRC therapy/prevention.

1.5 Ceramide: a lipid second messenger molecule

Ceramide belongs to the sphingolipid family and is considered as a biologically active molecule due to its role as lipid second messenger. Ceramide and other active sphingolipids are involved in the regulation of diverse cellular responses to exogenous stimuli (Spiegel, Foster *et al.*, 1996). A regulating role has been attributed to ceramide in many signalling pathways involving the cell cycle, differentiation, senescence and apoptosis. This is the main reason why ceramide action and the regulation of its production have recently attracted the scientific community attention. In major part, effects of ceramide are antagonistic to growth and survival (Ruvolo, 2001).

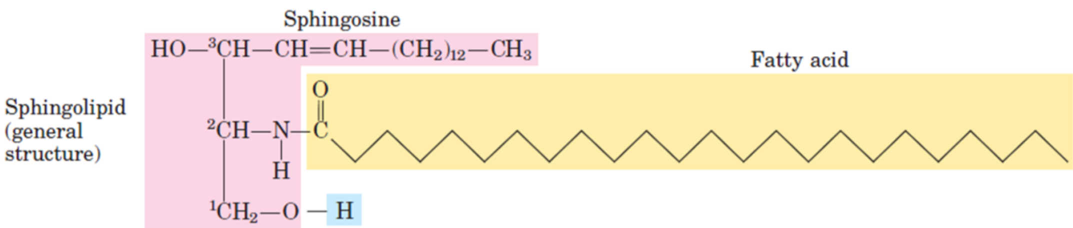


Figure 1.5 – Ceramide structure representation. Adapted from (Nelson and Cox, 2004).

Sphingolipids molecules are constituents of all eukaryotes as membrane cells components and they can be found in milk fat globule membranes, skin lamellar permeability barrier and lipoproteins (Schmelz, 2004). Their basic constitution is one molecule of the long-chain amino alcohol – sphingosine (or one of its derivatives) and a long-chain fatty acid. A polar head group is connected to the structure by a glycosidic or a phosphodiester linkage, according to the situation. The ceramide formation occurs when a fatty acid is attached in an amide linkage to the –NH2, on the C-2 (Figure 1.5), and this represents the structural origin of all sphingolipids (Nelson and Cox, 2004).

1.5.1 Ceramide synthesis: major biosynthetic pathways

Ceramide is the central point of sphingolipid metabolism. It serves as a crucial local of sphingolipid accumulation and it is a precursor for all major sphingolipids in eukaryotic cells. Synthesis of ceramide can occur through three major pathways: the *de novo* synthesis pathway, the sphingomyelinase (SMase) pathway and the salvage or sphingosine pathway (Reynolds, Maurer *et al.*, 2004), represented in Figure 1.6.

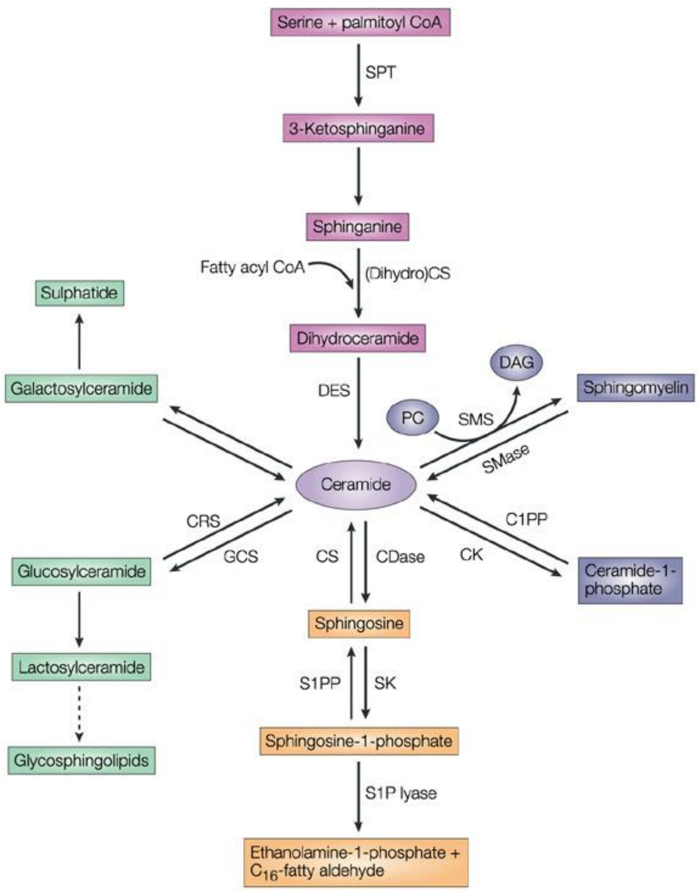


Figure 1.6 – Pathways of sphingolipid metabolism. Adapted from (Ogretmen and Hannun, 2004). The *de novo* pathway (pink); sphingomyelinase (SMase) pathway (blue); salvage pathway (orange); cerebrosides (green). Ceramidases, CDases; sphingosine kinases, SKs; sphingosine-1-phosphate, S1P; C1PP, ceramide-1-phosphate phosphatase; CRS, cerebrosidase; CK, ceramide kinase; CS, ceramide synthase; DAG, diacylglycerol; DES, dihydroceramide desaturase; GCS, glucosylceramide synthase; PC, phosphatidylcholine; S1PP, S1P phosphatase; SMS, sphingomyelin synthase; SPT, serine palmitoyltransferase.

1.5.1.1 The *de novo* synthesis pathway

The *de novo* pathway begins with the condensation reaction between the amino acid serine and palmitoyl-CoA resulting in 3-ketosphinganine formation. After that, the compound is reduced by the 3-ketosphingamine reductase enzyme, forming sphinganine and, by the action of ceramide synthase, dihydroceramide is formed. Finally, dihydroceramide is converted into ceramide, adding to it a trans-4,5 double bond, by the action of a desaturase (Merrill, 2002). In an alternate manner, this pathway may re-utilize sphingosine released after the degradation of more complex sphingolipids (Reynolds, Maurer *et al.*, 2004).

The *de novo* synthesis is believed to occur in the endoplasmic reticulum (ER) and probably in ER-associated membranes including the perinuclear membrane and mitochondria-associated membranes – MAMs (Hannun and Obeid, 2008).

The stimulation of the pathway can be achieved by drugs and ionizing radiation that normally results in a prolonged ceramide elevation (Liao, Haimovitz-Friedman *et al.*, 1999). Some chemotherapeutic agents can also enhance the *de novo* pathway stimulation; etoposide and daunorubicin are examples of them (Bose, Verheij *et al.*, 1995; Perry, Carton *et al.*, 2000) . Others inducers of apoptosis like the B-cell receptor, seem to have the same activating effect (Kroesen, Jacobs *et al.*, 2003). Obviously, the metabolic loading of either serine or palmitate can lead to ceramide production, as well as oxidized low-density lipoprotein - LDL (Kitatani, Nemoto *et al.*, 2002), cannabinoids (Gómez del Pulgar, Velasco *et al.*, 2002) and heat stress (Jenkins, Cowart *et al.*, 2002). Studies realized have largely implicated this pathway in mediating some effects of the inducers, described above, on stress responses and apoptosis (Kitatani, Idkowiak-Baldys *et al.*, 2008).

1.5.1.2 The sphingomyelinase pathway

Ceramide can be generated through the activation of sphingomyelinases (SMases), a special class of phospholipid phosphodiesterases, found in cell membranes. These enzymes are responsible for hydrolyzing sphingomyelin to originate ceramide and phosphocholine (Jones, Hannun *et al.*, 2005).

Different types of SMases exist, differentiated mainly by their optima (acid, neutral or alkaline) pH. The acid SMase (aSMase) has an optimum pH between 4.5 and 5.0 and was initially considered to be strictly located in the lysosome. Nevertheless, an isoform has been described to be present in vesicles near the plasma membrane and, under certain conditions, the aSMase could also relocate to the outer leaflet of the plasma membrane (Grassmé, Schwarz *et al.*, 2001; Hannun and Obeid, 2008). Furthermore, aSMase was shown to be secreted extracellularly (Schissel, Keesler *et al.*, 1998). In sum, there may be three types of aSMase: the acidic lysosomal aSMase (responsible for the sphingomyelin metabolism), the secretory aSMase (associated with inflammation and stress responses) and the receptor-activated aSMase (that translocates to the outer cell membrane, after activation by various cell surface receptors and hydrolyzes membrane sphingomyelin into ceramide).

Two neutral SMases (nSMases) have been identified: nSMase1 and nSMase2. Although both proteins are capable of converting sphingomyelin into ceramide, evidences are against a role for nSMase1 in regulating this reaction in cells (Tepper, Ruurs *et al.*, 2001). The nSMase2 has an optimum pH of 7.4 and appears to be localized to the inner leaflet of the plasma membrane (Hannun and Obeid, 2008).

The alkaline SMase (alk-SMase) has an optimal alkaline pH at 9 and its activity is dependent on bile salts (Hertervig, Nilsson *et al.*, 1997). This enzyme is expressed in the intestinal mucosa in many species and human bile too. In the intestinal tract, alk-SMase activity is superior in the jejunum and lower in the duodenum and colon, being responsible for the hydrolysis of dietary sphingomyelin (Duan, 2006).

Sphingomyelinase pathway is stimulated in response to cell treatment with TNF-α, Fas ligand or oxidative stress (Hannun, Luberto *et al.*, 2001; Marchesini and Hannun, 2004).

1.5.1.3 The salvage or sphingosine pathway

Another important mechanism for ceramide generation is the salvage pathway, in which sphingosine (produced from the metabolism of complex sphingolipids, mostly in the lysosome) is re-cycled to ceramide, localized then in the ER or associated membranes (Hannun and Obeid, 2008). Various enzymes are part of this pathway including ceramidases, (dihydro)ceramide synthase, SMases and probably glucocerebrosidase (acid-β-glucosidase).

This alternative *via* of ceramide generation is thought to be crucial for sphingolipid breakdown, in sphingolipid synthesis/turnover as well as in cellular signal transduction. These assumptions are based on the fact that the salvage pathway, leading to the re-regeneration of sphingolipids, has been estimated to contribute in 50% to 90% in sphingolipid biosynthesis (Tettamanti, Bassi *et al.*, 2003).

Salvage pathway is stimulated in response to cell treatment with TNF-α (Kim, Linardic *et al.*, 1991), Fas ligand (Brenner, Ferlinz *et al.*, 1998) or oxidative stress (Goldkorn, Balaban *et al.*, 1998).

1.5.2 Inhibitors of ceramide biosynthesis

Certain compounds and molecules have an inhibitory action upon ceramide synthesis and are able to stop those pathways at different levels. The same effect can be obtained using other techniques such as the knockdown of a particular enzyme of the pathway, or using microRNA. Some noteworthy inhibitors will be briefly described below.

Ceramide *de novo* synthesis can be inhibited at the serine palmitoyltransferase (SPT) level by an antifungal molecule called sphingofungin B (Zweerink, Edison *et al.*, 1992). Myriocin, an antifungal antibiotic, also stops the action of SPT. It was described as possessing immunosuppressant properties (Miyake, Kozutsumi *et al.*, 1995) and, after some developments, an analogue of this molecule was created – FTY720 (Adachi, Kohara *et al.*, 1995).

Fumonisins (Desai, Sullards *et al.*, 2002), originated by *Fusarium verticilioides* and *Fusarium moniliforme*; the fumonisins related AAL-toxins, produced by the fungus *Alternaria alternata* (Winter, Gilchrist *et al.*, 1996); australifungins (Mandala, Thornton *et al.*, 1995), micotoxins coming from *Sporormiella australis* are among the most studied inhibitors of ceramide synthase (CerS). Among all, fumonisin B_1 (FB_1) is the most representative member of this class of compounds. This molecule is a powerful inhibitor of CerS, acting in both *de novo* and salvage pathways of ceramide production (Kitatani, Idkowiak-Baldys *et al.*, 2008).

Inhibition can also happen in the SMase pathway, by cessing the activity of the different SMases. For example, desipramine is a tricyclic antidepressant that acts upon aSMase inducing its proteolysis (Elojeimy, Holman *et al.*, 2006). Several compounds have been described as inhibitors of nSMase. Manumycin A irreversibly inactive the nSMase (Arenz, Thutewohl *et al.*, 2001) while Scyphostatin has a reversible effect, but also reversibly inhibits the aSMase (Nara, Tanaka *et al.*, 1999; Arenz and Giannis, 2000). Sphingolactones, a new family of molecules, are also reported as irreversible inhibitors of the neutral SMase (Wascholowski and Giannis, 2006). During a high throughput screening on Mg^{2+} dependent nSMase, a compound called GW4869 was discovered and exhibited inhibitory properties against this enzyme (Delgado, Casas *et al.*, 2006).

1.5.3 Role of ceramide in vital cellular processes and apoptosis

Sphingolipid metabolism and, more particularly, ceramide have been implicated in many cellular processes. Ceramide has been seen as a coordinator of cellular stress responses, taking part of mechanisms as the cell cycle, differentiation, cell senescence and apoptosis.

During a growth phase, ceramide and phosphatidylcholine are converted into sphingomyelin (Hannun, 1994) and diacylglycerol (DAG), a product of this reaction, is a strong protein kinase C (PKC) activator (Nishizuka, 1995). This activation of PKC pathways by DAG sustain cell survival (Ruvolo, 2001). On the opposite, upon stress conditions, ceramide is produced by the breakdown of sphingomyelin (by SMases) (Hannun and Luberto, 2000). Sphingomyelin synthase appears to regulate DAG and ceramide possibly balancing the fate of cells between growth and apoptosis (Luberto and Hannun, 1998).

Some studies reported that differentiation of leukaemia cells happened when they were treated with ceramide and its analogues, imitating the effects of TNF-α, 1α, 25-dihydroxyvitamin D_3 and γ-interferon, on the cell line studied (Okazaki, Bielawska *et al.*, 1990).

Ceramide also demonstrated to be important when cells enter in the senescence phase, since its quantity increases (4-fold) in a human fibroblast cell line (Venable, Lee *et al.*, 1995), but much remains to be elucidated about the mechanisms behind those observations.

Autophagy is a vital procedure in a living cell and it has also been connected to ceramide. Some models have shown that ceramide-induced autophagy is a response to starvation, resulting from nutrient transporter downregulation (Peralta and Edinger, 2009). Moreover, ceramide-mediated macroautophagy resulted in up-regulation of beclin 1 and inhibition of protein kinase B - PKB (Scarlatti, Bauvy *et al.*, 2004). Besides, after proving that ceramide signalling occurs through the activation of Akt/PKB, upstream of mTOR, it was shown that ceramide stimulates JNK1 to phosphorylate Bcl-2, after dissociation of beclin 1 (Pattingre, Tassa *et al.*, 2005; Pattingre, Bauvy *et al.*, 2008; Pattingre, Bauvy *et al.*, 2009).

Over the years, ceramide role in apoptosis has attracted many attentions due to the finding of its pro-apoptotic role.

Environmental stresses and several cytokines (that trigger apoptosis) such as TNF, CD95/Fas/APO-1, ultraviolet-C, ionizing radiation, oxidative stresses, heat shock, chemotherapeutic agents, among others, appear to quickly induce ceramide production (Pettus, Chalfant *et al.*, 2002). In fact, it is important to refer that the doses of these agents necessary to produce ceramide are really close to those required to induce apoptosis (Kolesnick and Kronke, 1998). Also, that the elevation of cellular concentration of ceramide, in response to those agents, occurs prior to the execution phase of apoptosis (Birbes, El Bawab *et al.*, 2002). Another interesting notion about cell-permeable ceramide and analogues is that its exogenous administration induces apoptosis in multiple cell types (Cifone, De Maria *et al.*, 1994; Jarvis, Kolesnick *et al.*, 1994). Furthermore, apoptosis can be inhibited by stopping ceramide production and cells that cannot generate ceramide are unable of undergoing programmed cell death (Selzner, Bielawska *et al.*, 2001). Otherwise, in cancer cells, apoptosis can be induced by the elevation of ceramide levels, using inhibitors of ceramide synthesis or by externally adding cell-permeable ceramide analogues (Selzner, Bielawska *et al.*, 2001; Siskind, 2005).

Notwithstanding all the studies that have been made, in order to understand by which process ceramide generation can trigger apoptosis, the subject remains not fully understood. Some direct and probable targets of ceramide action have been found (Figure 1.7) and, among them, some that combine ceramide formation with downstream inducers of programmed cell death are the following ones: cathepsin D, ceramide activated protein kinases (CAPK) and serine/threonine protein phosphatase 1 (PP1) and serine/threonine protein phosphatase 2A (PP2A) (Heinrich, Wickel *et al.*, 2000; Pettus, Chalfant *et al.*, 2002). Additionally, ceramide and its metabolism might be associated to LMP induction and the activation of the lysosomal cathepsin D (Benes, Vetvicka *et al.*, 2008).

As observed in Figure 1.7, many pathways are involved in ceramide-induced apoptosis. Ceramide action towards mitochondria has been studied. Ceramide activated protein phosphatases (CAPP) are responsible for dephosphorylating Bcl-2 and Bcl-2 kinase PKCα, both acting on this organelle. As the Bcl-2 function is to inhibit the cytochrome *c* release, it has been suggested that mitochondrially generated ceramide leads to cytochrome *c* release and, in consequence, to programmed cell death (Kagedal, Johansson *et al.*, 2001; Pettus, Chalfant *et al.*, 2002).

Others interactions have been taken in consideration. Proteins p53 and Rb (retinoblastoma protein) seem to act along with ceramide generation, upstream of ceramide production for p53 and downstream for Rb that is dephosphorylated by CAPPs. In cell lines where agonists do not seem to trigger programmed cell death, Rb associated with ceramide is involved in cell cycle arrest, which could explain the reason why sometimes apoptosis is activated and, in other situations, only cell cycle arrest is observed (Alberts, Thorburn *et al.*, 1993; Dbaibo, Pushkareva *et al.*, 1995).

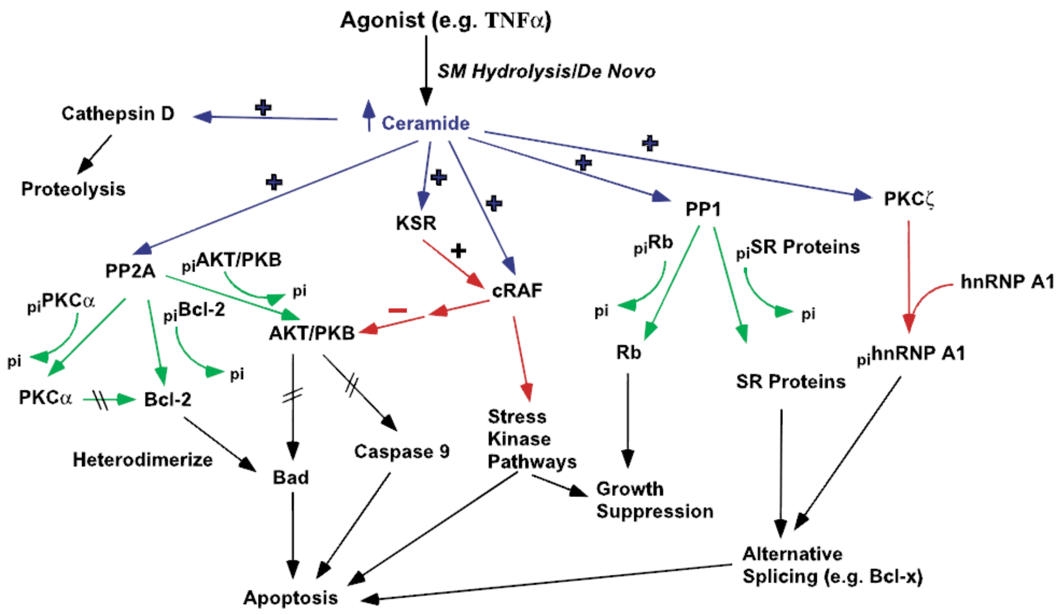


Figure 1.7 – Ceramide effector pathways relevant to apoptosis. Adapted from (Pettus, Chalfant *et al.*, 2002).

Upon stimulation (by FAS or chemotherapy, for example) apoptosis can be induced by the action of cathepsin D, which could, in turn, have been triggered by ceramide (Heinrich, Wickel *et al.*, 2000). The same outcome can be obtained by stimulation of TNF receptors. Endosomes are formed after internalization and then fused with lysosomes generating multilamellar bodies, contacting directly with lysosomal aSMase. The TNF-receptor activates aSMase, releasing intra-vesicular ceramide that binds cathepsin D. The last one translocates to the cytoplasm where it triggers cell death via Bid, Bax and Bak molecules (Schneider-Brachert, Tchikov *et al.*, 2004).

Another mechanism by which ceramide-induced apoptosis could be achieved has been described. Thus, death receptors CD95 and DR5, in an activated state, trigger the translocation of aSMase to the extracellular leaflet of the plasmatic membrane, which leads to the formation of ceramide-enriched membrane platforms (sphingomyelin transformed into ceramide by aSMase). This leads to the re-organization of CD95 and DR5 in small clusters. Despite not being completely understood this process has revealed to be necessary for an efficient signalling, of these receptors, leading to programmed cell death (Carpinteiro, Dumitru *et al.*, 2008).

Many "routes" seem to lead to apoptosis in ceramide behalf, but more investigation in that field is needed to better understand in which precise conditions and by what processes programmed cell death is activated.

1.5.4 Role of ceramide in colorectal carcinoma

As mentioned previously, resistance to cell death is one of the cancer hallmarks. Ceramide and its analogues have proved that they were capable of inducing cell death, namely apoptosis, in several types of cells, including CRC. It is known that the levels of ceramide in CRC are lowered in 50% and, also, that the application of ceramide and analogues induce apoptosis *via* caspases and the release of cytochrome *c* (Selzner, Bielawska *et al.*, 2001).

In CRC cell lines, such as HT-29 and HTC-116, ceramide and sphingoid bases caused cell death (Ahn and Schroeder, 2002). In addition, C2-ceramide treatment of cells that harbour the *APC* gene induced apoptosis through ceramide, mediated *via* a reduction in the levels of this gene (Jaiswal and Narayan, 2004).

Ceramide has the potential of being used as a treatment to CRC and other forms of tumours. Increasing intracellular ceramide levels could be used to reach growth arrest and activate apoptosis in malignant cells. In contrast, diminishing ceramide levels would work in favour of proliferation (Kolesnick, 2002).

As aforementioned, cellular stress increase ceramide levels in cells, in consequence chemotherapeutic agents (radiotherapy, etoposide, vincristine, fenretinide, irinotecan, etc.) also have the same effect (Senchenkov, Litvak *et al.*, 2001). Maybe the use of strategies interfering with the inhibition of ceramide generation could sort some effect, as it happened with the ceramidase inhibitor B13 for example, that raised the cellular content in ceramide, led to the rapid release of cytochrome *c*, the activation of caspase 3 and specifically induced cell death of cancer cells, without affecting healthy ones, *in vivo* (Selzner, Bielawska *et al.*, 2001). In experiences realized in an *in vivo* model (nude mice), B13 also prevented the growth of aggressive forms of human colon cancer. Cancer cells were injected in nude mice and some weeks after the injection tumour growth was determinate; the majority of animals that received a B13 treatment remained completely cancer free (Selzner, Bielawska *et al.*, 2001). Interrupt the S1P synthesis (pro-survival molecule) could also be an interesting method to test, as it could help triggering ceramide-mediated programmed cell death (Reynolds, Maurer *et al.*, 2004).

A lot of strategies to fight cancer could be used but betting in mechanisms that could lead to the specific death of cancer cells is the major objective. Ceramide has reunited good evidences to become a part of such strategies but, in the meantime, much more investigation need to be done to really understand how this secondary messenger could be manipulated in order to achieved such goal.

2

Objectives

2.1 *Rationale* of the project

Colorectal carcinoma is a common form of tumour and one of the most impacting worldwide. *Propionibacteria* are a group of microorganisms that live in the human organism and as a result from their metabolism short-chain fatty acids are produced, among them are acetate, butyrate and propionate. In 2002, Jan and co-workers demonstrated that SCFAs were able to induce cell death in colorectal carcinoma cells by apoptosis, without affecting healthy cells. It was also suggested that mitochondria would have a role to play in this cell death pathway (Jan, Belzacq *et al.*, 2002). More recently, similar results were obtained by other research groups, linking SCFA-induced apoptosis to environmental factor such as the pH (Lan, Lagadic-Gossmann *et al.*, 2007) and unveiling the deadly action of SCFAs *in vivo* (Lan, Bruneau *et al.*, 2008).

Studies carried using the yeast *Saccharomyces cerevisiae* demonstrated that acetic acid could trigger the programmed cell death in yeasts associated with mitochondria and the release of cytochrome *c* (Ludovico, Sousa *et al.*, 2001; Ludovico, Rodrigues *et al.*, 2002). Furthermore, apoptosis induced by acetic acid was also found to be connected to the Pep4p (orthologue of mammalian cathepsin D) translocation from the vacuole to the cytosol. The mechanisms that occur in yeasts are often similar to those happening in more complex organisms, but is acetic acid/acetate able to determine such events in mammalian cells? Recent experiments realized in our laboratory demonstrated that apoptosis was activated by acetate in CRC cell lines and that same compound led to lysosomal membrane permeabilization (LMP) and release of cathepsin D to the cytosol (Marques, Oliveira *et al.*, 2012 ; submitted).

Sphingolipids, namely ceramide, play a role in a variety of cellular responses including cell growth, differentiation, senescence and cell death. Moreover, ceramide proved to have a determinant role in the induction of apoptosis in colorectal carcinoma cells (Ahn and Schroeder, 2002) and this molecule and its metabolism might be associated to LMP induction and the activation of the lysosomal cathepsin D (Benes, Vetvicka *et al.*, 2008).

The involvement of ceramide as a mediator of the acetate-induced cell death, in CRC cell lines, has not been investigated. As aforementioned, it has been described that acetate induce apoptosis in CRC cells and that ceramide also had a role on the process. Therefore, the possibility of the programmed cell death triggered by the SCFA being associated to ceramide metabolism was a good relation to be exploited. Certainly, understanding this process will lead to a new therapeutic strategies and help improving existent ones.

2.2 Objectives

Ceramide, as an important secondary messenger, and its metabolism seem to be interconnected to many major cellular processes. Evidences collected over the years, by our research group and other teams, point that this relation might be also present in acetate-induced apoptosis observed in colorectal carcinoma cell lines.

Consequently, the objective of this project was to understand the role of ceramide in acetate-induced cell death in CRC cells. More particularly, the aim was centred in elucidating the involvement of ceramide and its synthesis pathways in the activation of apoptosis, triggered by acetate in colorectal carcinoma cell lines.

Bearing in mind this general goal, we specifically aimed at:

1. Establish the half maximum inhibitory concentration (IC_{50}) of acetate, and determine the sub-lethal concentration of the ceramide metabolism inhibitors, for each cell line used.
2. Study the role of the different biosynthesis pathways of ceramide in acetate-induced cell death in CRC cells by assessing the effect of inhibiting the sphingomyelinase (SMase) pathway, the salvage pathway and the *de novo* synthesis pathway of ceramide, using pharmacological inhibitors of specific steps of ceramide metabolism.

3

Material and Methods

3.1 Cell culture: cell lines and culture conditions

To fulfil the objectives of the present work, two different cell lines were used: HCT-15 (CCL-225) and RKO (CRL-2577), from the American Type Culture Collection (ATCC), that were kindly provided by the Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP). Both cell lines were originated from colorectal carcinoma, but harbour different genetics backgrounds.

HCT-15 cell line was derived from a human colorectal adenocarcinoma (Dukes' type C) and was established by Jean C. Hager, from solid tumour material obtained during surgery and before drug treatment (Tibbetts, Chu *et al.*, 1977; Dexter, Barbosa *et al.*, 1979). These cells harbour several mutations, including a *KRAS* ^{G13D} and a *TP53* mutation (Küntzer, Eggle *et al.*; Preto, Figueiredo *et al.*, 2008). HCT-15 cells were grown in RPMI 1640 (with stable glutamine) medium (PAA, Austria), supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS; Sigma, USA) and 1% (v/v) penicillin-streptomycin (5,000 Units/mL of penincilin and 5,000 µg/mL of streptomycin; Gibco, Invitrogen).

RKO cell line was originally extracted from a primary colon carcinoma and was developed by Michael Brattain (Brattain, Levine *et al.*, 1984). RKO harbours five main gene mutations, including a *BRAF* ^{V600E} mutation (Küntzer, Eggle *et al.*; Preto, Figueiredo *et al.*, 2008). These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; PAA, Austria), supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS; Sigma, USA), 1% (v/v) penicillin-streptomycin (5,000 Units/mL of penicillin and 5,000 µg/mL of streptomycin; Gibco, Invitrogen) and 2% (v/v) of sodium bicarbonate 7.5% (Gibco, Invitrogen).

Both cell lines were grown and maintained in 25 cm^2 tissue culture flasks (TPP), in a humidified incubator at 37°C, containing 5% of carbon dioxide (CO_2). Renewal of the medium was effectuated 1 or 2 times per week, and the cells were sub-cultured when the confluence reached 80%-100%.

The sub-culture was initially performed by removing the medium, contained in the culture flask, and cells were washed twice with 1mL of PBS (phosphate buffered saline) 1x solution. In order to detach the adherent cells from the flask, they were incubated with 0.5 mL of a 0.05% trypsin-0.02% EDTA solution (Gibco, Invitrogen), at 37°C, in a 5% CO_2 atmosphere, for a few minutes (depending on the cell type), to help the reaction. After cell detachment, 4.5 mL of fresh and supplemented medium were added to neutralize the trypsin effect and obtain a cell suspension. Cells were then seeded in 25 cm^2 flasks at the desired density, using fresh medium.

3.2 MTT reduction assay

The MTT reduction assay is one of the most common techniques used in cell biology for the quick assessment of cell viability and proliferation (Mosmann, 1983). It is also widely used as an assay to determine compounds cytotoxicity, for example, the chemosensitivity of a particular cell type to anticancer drugs (Carmichael, DeGraff *et al.*, 1987).

The method's principle consists in the reduction of the tetrazolium salt thiazolyl blue, also termed **MTT**, from **m**ethyl-**t**hiazolyl-**t**etrazolium (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), in formazan, a water insoluble and (purple) coloured compound, which can be spectrophotometrically quantified (Figure 3.1). This reaction only occurs in cells with metabolic activity, and the amount of formazan crystals formed is directly proportional to the quantity of viable cells (Belyanskaya, Manser *et al.*, 2007).

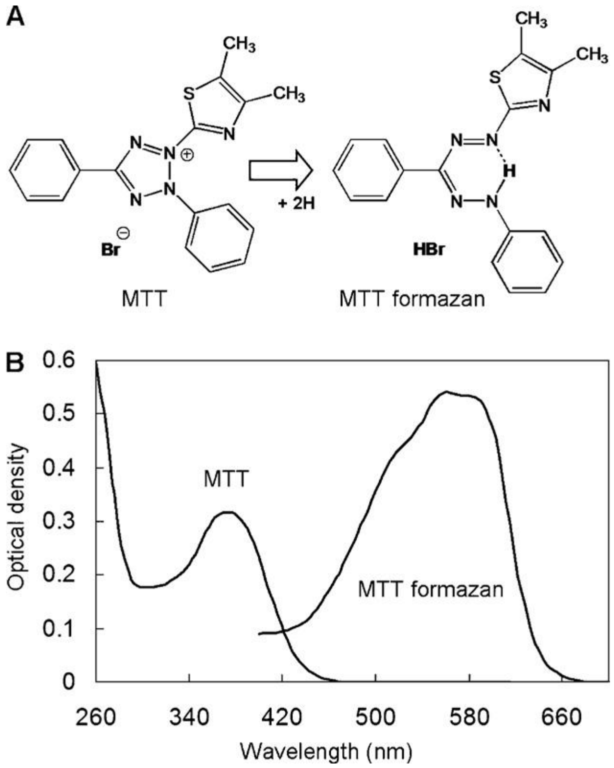


Figure 3.1 – (A) Representation of the chemical structure of MTT and its reduced formazan product; **(B)** absorption spectra of MTT and MTT formazan, in distilled water and sunflower oil, respectively, both at the same concentration (0.016 mg/ml). Adapted from (Stockert, Blázquez-Castro *et al.*, 2012).

Despite of being widely used, the mechanism of MTT reduction is poorly understood, neither the subcellular localization, nor the biochemical events are fully known. It was initially assumed that the reduction of MTT was accomplished by mitochondrial enzymes, being MTT reduction supported by the specific substrate succinate, on active mitochondria of living cells, as demonstrated by Slater and co-workers, using rat liver homogenates (Slater, Sawyer *et al.*, 1963). Nevertheless, other studies showed that this reaction could also be carried by nonmitochodrial enzymes, such as dehydrogenases or flavin oxidases, and that NADH or NADPH are better substrates in MTT reduction than succinate (Liu, 1999). MTT reduction could be carried in the cytoplasm by NADH and by dehydrogenases associated to the endoplasmic reticulum (Berridge, Tan *et al.*, 1996), in the plasma membrane (Bernas and Dobrucki, 2000) and endosome/lysosome vesicles (Bernas and Dobrucki, 2002).

It was also postulated that MTT is non membrane permeable and, therefore, it enters the cells by endocytosis. Furthermore, its reduction would be performed by a *N*-ethylmaleimide-sensitive flavin oxidase and the intracellular formazan granules formed would accumulate in endosomal or lysosomal vesicles and ultimately be transported outside of the cell by exocytosis, under the aspect of needle-like crystals (Liu, Peterson *et al.*, 1997). However, other hypothesis are supported to explain the efflux of formazan, such as physico-chemical interactions at molecular level (Diaz, Melis *et al.*, 2007). Despite MTT reduction mechanism still remaining partially unclear, it is a valuable tool to determine cellular viability.

To perform MTT assays, cells were seeded in 96-well plates at a final cellular density of $1x10^4$ cells/well for HCT-15 cell line and $7,5x10^3$ cells/well for RKO cell line, with a final volume of 100 µL per well. Cells were incubated overnight, between 16h to 24h, in a 37°C incubator, with a 5% CO_2 and humidified atmosphere, to promote cellular adhesion to the plate. After that, the intended treatment was applied to the cells (conditions were performed in triplicate). For this purpose, the medium was removed, allowing the removal of dead cells, and replaced with fresh medium in which the desired compounds were added. Cells were maintained in the incubator, in the same conditions as cited above, during 48h.

At this time point, 10 µL of MTT solution (Sigma, USA), prepared at a concentration of 5 mg/mL in PBS 1x (pH 7.4) and maintained in the dark, was added to each well, resulting in a final concentration of 0.5 mg of MTT per mL. The 96-well plate was wrapped with aluminium foil and re-incubated for a 4 hours period, allowing the metabolism of MTT and formation of visible formazan crystal. These crystals were solubilised by adding a solubilisation solution (89% isopropanol, 10% Triton-X and 1% of HCl 37%) and each well was ressuspended. After all crystals were completely dissolved the absorbance was read in a microplate reader (SpectraMax Plus^{384} Microplate Reader – Molecular Devices) at a wavelength of 570-690nm (690nm reference value).

When seeded in 24-well plates, the protocol used was slightly different. A final cellular density of 2,5x10^{4} and 7x10^{4} cells/well was used for RKO and HCT-15 cell lines, respectively, with a final volume of 1 mL per well. After being seeded, cells were incubated and the desired treatment was applied in the same conditions as described above. At the required time point (48h), cell medium was removed and cells were rinsed, once, with 1 mL of sterile PBS 1x and 0.5 mL of MTT solution (0.5 mg/mL in PBS 1x pH 7.4) was added to each well (this solution was prepared just before its usage and maintained in the dark). Following that, the cell culture plates (wrapped in aluminium foil) were incubated in a 37°C incubator, with a 5% CO_2 and humidified conditions, for 2 hours.

After allowing the formation of formazan crystals, 0.5 mL of acidic isopropanol (0.04 M HCl in absolute isopropanol) were added to the wells, in order to dissolve the formazan crystals. The plates, always protected from light, were placed in an orbital shaker for 30 minutes to help the dissolution of the formazan crystals. With a micropipette, each well was homogenised, to solubilise the remaining crystals, and 0.2 mL were transferred to a 96-well cell culture plate. Absorbance was also read in a microplate reader (SpectraMax Plus^{384} Microplate Reader – Molecular Devices) at a wavelength of 570-690nm (690nm reference value).

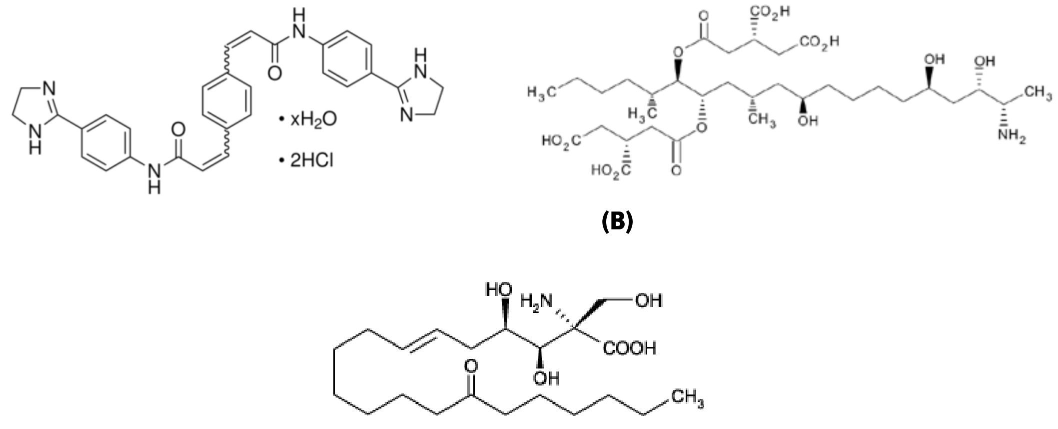
The results obtained were expressed as a percentage of cell viability in relation to the negative control (untreated cells).

3.3 Cell treatment with acetate and ceramide pathway inhibitors: GW4869, fumonisin B_1 and myriocin

In the attempt to verify if ceramide is involved in acetate-induced apoptosis, HCT-15 and RKO cell lines were pre-treated with inhibitors of the ceramide pathways before induction of cell death by incubation with acetate and in the absence or presence of acetate (co-incubation). The analyses were performed using 96-well plates and the cellular viability was determined by MTT assay.

Three different inhibitors were used (Figure 3.2). GW4869 ($C_{30}H_{28}N_6O_2$ · 2HCl · xH2O; Sigma, USA) is a drug that impedes the action of neutral sphingomyelinase (nSMase), interfering in the sphingomyelinase (SMase) pathway; fumonisin B_1 - FB_1- ($C_{34}H_{59}NO_{15}$; Enzo Life Sciences), is an inhibitor of ceramide synthase (CerS), acting in the salvage pathway of ceramide biosynthesis and myriocin ($C_{21}H_{39}NO_6$; Enzo Life Sciences) inhibits the action of serine palmitoyltransferase (SPT), at the *de novo* synthesis pathway of ceramide biosynthesis (Figure 3.3).

GW4869 was dissolved in dimethyl sulfoxide (DMSO) at a 20 mM stock concentration and stored at -20°C, while fumonisin B_1 was dissolved in sterile ultra-pure water at a 20 mM stock concentration and also stored at -20°C. To determine the higher concentration that would have less toxic effect in the studied cell lines, a range of concentrations was tested for each inhibitor.



(A) **(B)** **(C)**

Figure 3.2 – Representation of the molecular structure of GW4869 **(A),** fumonisin B_1 **(B)** and myriocin **(C).**

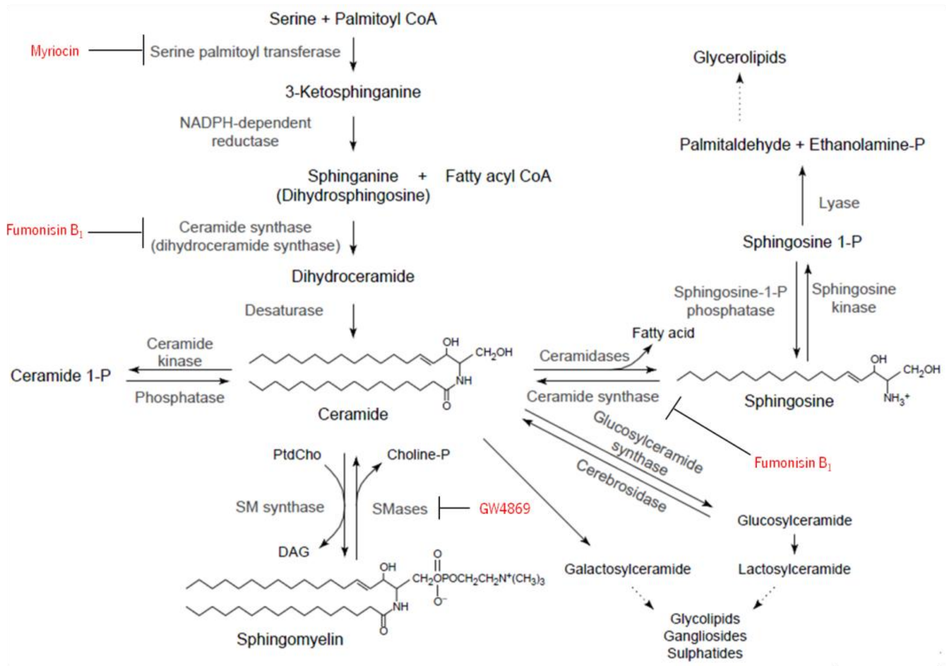


Figure 3.3 – Ceramide biosynthesis pathways and inhibitors: GW4869, fumonisin B1 and myriocin (red). Adapted from (Hannun and Luberto, 2000).

To test the concentration range of the two inhibitors a solution of GW4869 50 µM and of FB_1 100 µM were diluted in supplemented medium and used to obtain the solutions with the selected concentrations. Cell lines were pre-incubated and co-incubated with each concentration of inhibitors (GW4869: 5, 10, 20 and 50 µM; FB_1: 1, 5, 10, 15, 20, 40, 60 and 100 µM, for both cell lines). The concentration of GW4869 and fumonisin B_1 were chosen according to their effect on cell's viability, i.e. the higher concentration that had a lesser toxic outcome was chosen.

The pre-incubation consists in the medium removal and addition of fresh medium with the inhibitors to each well, during 30 minutes for GW4860 and 2 hours for fumonisin B_1. Then, the medium containing the drugs was replaced by fresh medium for 48h. In the co-incubation, medium containing the inhibitor was added to each well during 48h.

All the optimizations for myriocin ($C_{21}H_{39}NO_6$; Enzo Life Sciences, Figure 3.2) had already been done by our research group (Silva, 2012 ; Master thesis). The inhibitor was dissolved in DMSO and a stock solution with 25 mM was prepared and stored at -20°C.

Following the determination of the adequate concentrations of inhibitors, they were tested in combination with sodium acetate ($CH_3COONa.3H_2O$; Panreac Quimica SA, Spain), which from now on we will refer to as acetate. The concentration of acetate which is lethal to 50% of the cells, the half maximal inhibitory concentration (IC_{50}), was determined in 24 and 96-well plates, for both cell lines, using the MTT assay. The following concentrations of acetate were tested: 70, 100, 120, 140 and 200 mM, in 24-well plates, and two additional concentrations, 280 and 400 mM, in 96-well plates, for HCT-15 cell line; and 50, 70, 110, 140 and 220 mM in 24-well plates and 110, 125, 140, 180, 220, 300, 400 mM of acetate were tested in 96-well plates, for RKO cell line.

Table I – Concentrations of acetate used in the MTT assays.

	[Acetate] (mM)			
	HCT -15		RKO	
	24-well plate	96-well plate	24-well plate	96-well plate
IC_{50}	100	206	74	140
intermediate IC_{50}	150	309	111	210
2 x IC_{50}	200	412	148	280

Once the IC_{50} values for acetate were established (Table I) cell viability was assessed by the MTT assay, in both cell lines, after co-incubation with 5 μM, 10 μM and 150 nM of GW4869, fumonisin B_1 and myriocin respectively, and with acetate for 48h at concentrations corresponding to the IC_{50}, intermediate IC_{50} or 2 x IC_{50} values.

3.4 Statistical analysis

The data presented are, at least, from two independent experiments. Data are expressed as mean ± SEM or mean ± SD. The results were analyzed using one-way ANOVA, followed by a Dunnet's post test, when comparing the control and treated cells from one cell line. Differences were considered statistically significant when $p \leq 0.05$. All the analyses were performed using GraphPad PRISM software.

4

Results

Changes in intracellular ceramide levels have been linked to modulation of apoptosis in mammalian cells. Many inducers of ceramide accumulation are known to induce apoptosis and growth suppression and changes in ceramide cellular contents occur prior the execution phase of apoptosis (Birbes, El Bawab *et al.*, 2002). It is known that, in cancer cells, apoptosis can be triggered by the increase in ceramide levels, using inhibitors of ceramide synthesis or externally employing cell-permeable ceramide analogues (Selzner, Bielawska *et al.*, 2001; Siskind, 2005).

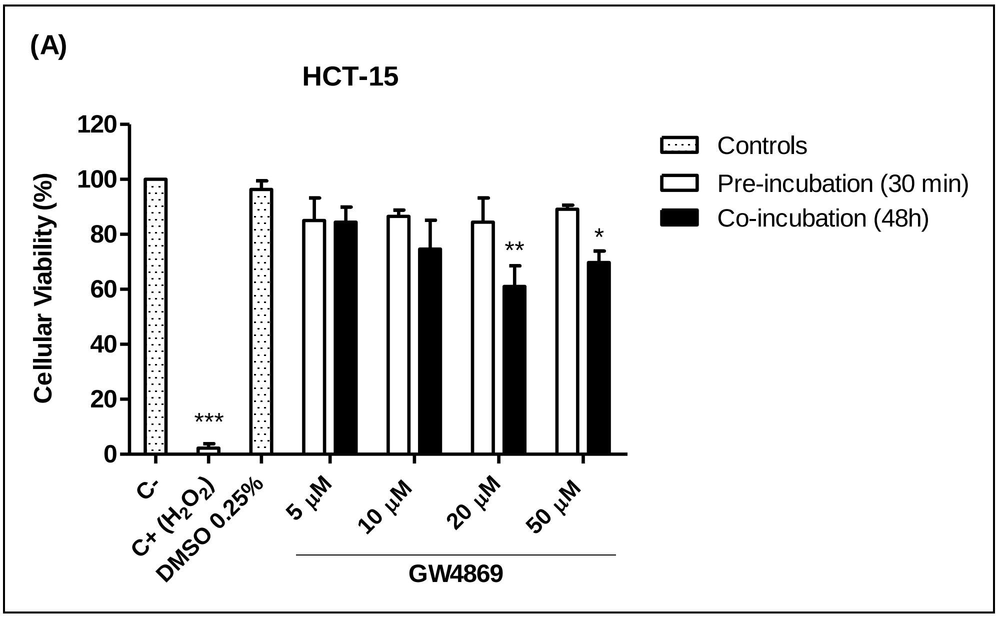
Our goal was therefore to characterize the contribution of enzymes involved in ceramide metabolism to apoptotic cell death induced by acetate in CRC cells. For this purpose we used different pharmacological inhibitors of specific steps of ceramide metabolism. It was therefore necessary to determine, in a first approach, the sub-lethal concentration of the inhibitors (section 4.1) as well as the half maximum inhibitory concentration of acetate (IC_{50}) (section 4.2) for the two CRC cell lines selected (HCT-15 and RKO). The role of ceramide metabolism on loss of cell viability induced by acetate was then assessed by determining the effect of ceramide inhibitors at the concentrations selected, alone or in combination, on the loss of cell viability induced by exposure to the IC_{50}, an intermediate concentration and 2 x IC_{50} of acetate.

4.1 Optimization of different concentrations of ceramide pathway inhibitors in CRC cell lines

The first part of the project was to test the ceramide inhibitors, GW4869 and fumonisin B_1 (FB_1), on HCT-15 and RKO cell lines (Figure 4.1 and 4.2), to observe their effect on cellular viability and define an adequate concentration of each one to be used later in combination with acetate. Several concentrations of GW4869 and FB_1, in the micromolar (μM) range, were tested, according to what was already previously described in other studies (Luberto, Hassler *et al.*, 2002; Marchesini, Luberto *et al.*, 2003; Kouadio, Mobio *et al.*, 2005; Cianchi, Cortesini *et al.*, 2006; Nagata, Partridge *et al.*, 2006).

The cell lines used in this study were pre-incubated 30 minutes with GW4869 or 2h with FB_1, or co-incubated with the inhibitors for a period of 48 hours.

The highest concentration of GW4860 (50 μM), reached a final concentration of 0.25% DMSO, higher than the limit normally used of 0.1%, thus it was tested to see an eventual effect of DMSO on cellular viability on its own. As it can be seen on Figure 4.1, no significant difference was found between treatment with 0.25% of DMSO and untreated cells, on both cells lines. For that reason, the conditions tested were compared to untreated cells - negative control (C-).



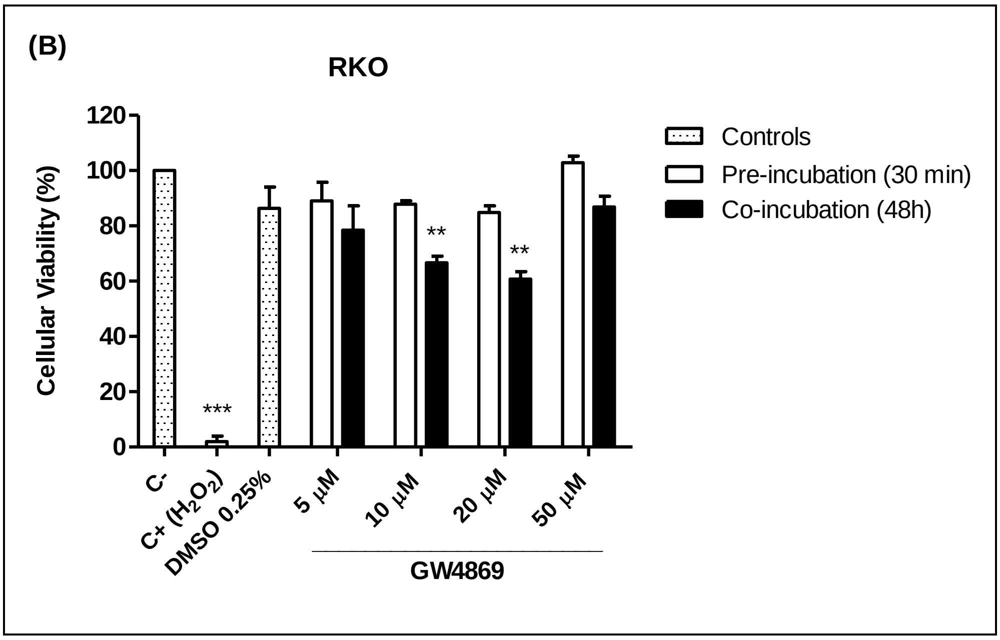


Figure 4.1 – Effect on cellular viability of different concentrations of GW4869 on HCT-15 **(A)** and RKO **(B)** cell lines, by MTT assay. Cells were seeded at a density of $1x10^4$ cells/well (A) and $7,5x10^3$ cells/well (B), in a 96-well cell culture plate. Different concentrations of GW4869 were incubated during 30 min (pre-incubation) and 48h (co-incubation). Negative control (C-) corresponds to cells incubated with fresh complete medium and the positive control (C+) corresponds to cells treated with 500 µM (A) and 1,5 mM (B) of hydrogen peroxide (H_2O_2), for HCT-15 and RKO cell lines respectively. The percentage of viability was calculated relatively to the value of absorbance obtained for the negative control. For each bar, mean ± SEM for two independent experiments (n=2) is represented. * $p \leq 0.05$; ** $0.01 \leq p \leq 0.001$; *** $p \leq 0.0001$, compared to control cells (C-), since the DMSO highest dose used revealed no significant differences compared to untreated cells (C-).

The inhibitor of nSMase (GW4869) seemed to have a similar effect on HCT-15 and RKO cell lines, as the percentages of viability are approximately the same. On a general view, the viability tended to diminish as the concentration of the drug increased, either in pre or co-incubation, except on the highest concentration (50 μM), where the percentage of viability slightly increased, especially with the RKO cell line.

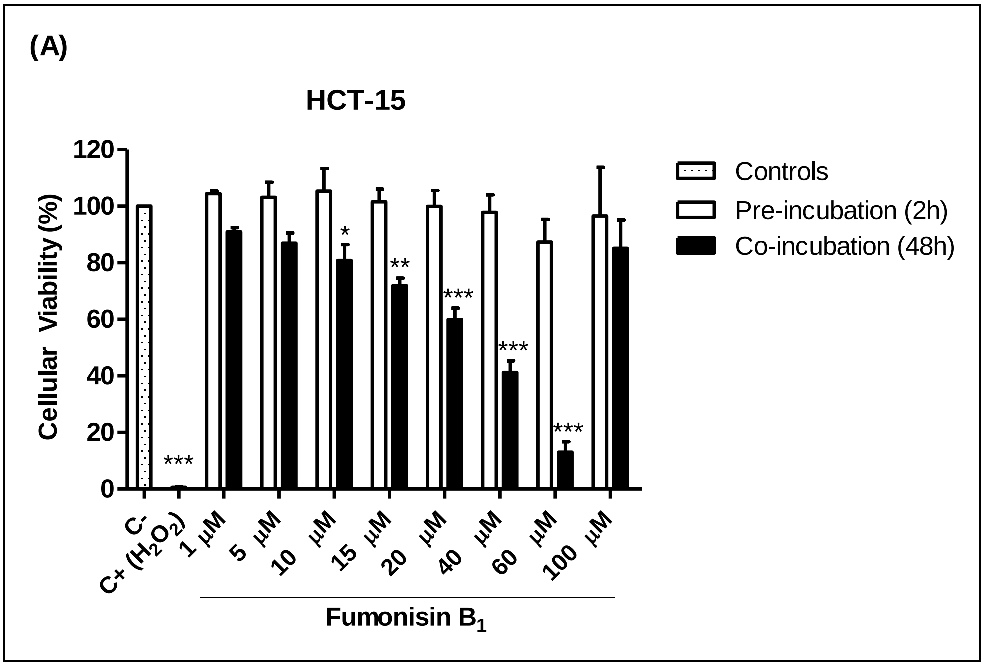
In the pre-incubation, no important changes in cell viability were verified. When cells were co-incubated with 20 μM of GW4869, a significant alteration in viability of cells in this condition was observed. The cellular viability also decreased when cells were treated with 50 μM of GW4869, although the difference was only visible in HCT-15 cells.

The behaviour of RKO cells pre-incubated with GW4869 (Figure 4.1-B) was similar to that observed in HCT-15. In the co-incubation condition, a concentration of 10 μM of GW4869 was sufficient to significantly alter the value of viable cells but, when the cell line was treated with a concentration of 50 μM of GW4869 the percentage of cellular viability increased again.

Fumonisin B_1, an inhibitor of the ceramide synthase (CerS), had a different impact than GW4869, on the cell lines studied (Figure 4.2).

For the HCT-15 cell line there were no significant alterations of the cellular conditions, when cells were pre-incubated with FB_1 (Figure 4.2-A). Concerning the co-incubation, a decrease on cell viability started to be observed at a concentration of 10 μM of FB_1. Thereafter, the decrease on cellular viability was proportional to the increase in the concentration until 60 μM of FB_1.

The same pattern can be seen on RKO cell line (Figure 4.2-B). There was no main alteration on the viability of the cells, regarding the pre-incubation but, on the opposite, the co-incubation led to a significant loss in RKO cellular viability (when the concentration of this drug reached 40 μM), but not as marked as it happened with HCT-15 cells. Like it was observed with the inhibitor of nSMase (GW4869), the highest concentration of FB_1 tested (100 μM), on both cell lines, did not lead to significant changes in cellular viability, either in pre-incubation or co-incubation.



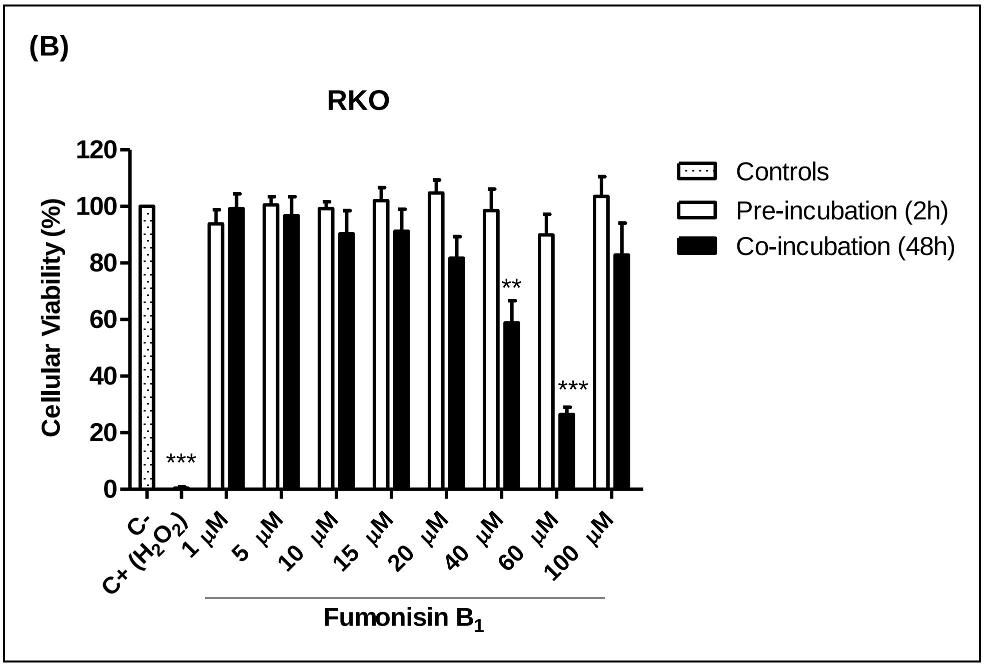


Figure 4.2 – Effect on cellular viability of different concentrations of fumonisin B_1 (FB_1) on HCT-15 **(A)** and RKO **(B)** cell lines, by MTT assay. Cells were seeded at a density of $1x10^4$ cells/well (A) and $7,5x10^3$ cells/well (B), in a 96-well cell culture plate. Different concentrations of FB_1 were incubated during 2h (pre-incubation) and 48h (co-incubation). The negative control (C-) corresponds to cells incubated with fresh complete medium and the positive control (C+) corresponds to cells treated with 500 µM (A) and 1,5 mM (B) of hydrogen peroxide (H_2O_2), for HCT-15 and RKO cell lines respectively. The percentage of viability was calculated relatively to the value of absorbance obtained for the negative control. For each bar, mean ± SEM for three independent experiments (n=3) is represented. * p≤0.05; ** 0.01≤p≤0.001; *** p≤0.0001, compared to control cells (C-).

4.2 Determination of acetate half maximum inhibitory concentration (IC_{50}) for HCT-15 and RKO cell lines

The IC_{50} of acetate was determined on 24 and 96-well cell culture plates, using the MTT assay. HCT-15 cells were seeded at a density of $7x10^4$ and $1x10^4$ cells/well in 24 and 96-well cell culture plates, respectively. For RKO, cells were seeded at a density of $2,5x10^4$ and $7,5x10^3$ cells/well in 24 and 96-well cell culture plates, respectively. After the total adherence of the cells, acetate treatment was applied, consisting in a wide-ranging set of concentrations tested for both type of cell culture plate. At the defined time point, 48h, MTT assay was performed.

As expected the results obtained showed that cellular viability decreased with increased concentrations of acetate in both cell lines (Figure 4.3). RKO cells seemed to be more vulnerable to acetate, as lower concentrations were needed to reach the half maximum inhibitory concentration comparing with HCT-15 cell line.

Looking at the graph for HCT-15 cells (Figure 4.3-A), it can be visualized that the acetate concentrations used with 96-well plate assays were higher than those used in 24-well plate assays. The 70 mM concentration tested in 24-well plates was already cytotoxic for HCT-15 cells and the other concentrations tested (100, 120, 140 and 200 mM) led to a gradual decrease of cell viability. Under these conditions the IC_{50} of acetate was estimated to be 100 mM in 24-well plates. For the 96-well plates we could observe that the first four concentrations tested (70, 100, 120 and 140 mM) did not alter in a noteworthy way the cellular viability, leading to what could be called a “plateau”. When 200 mM of acetate was tested cellular viability dropped significantly, becoming very low when cells were treated with higher concentrations. The IC_{50} determined for HCT-15 cells in 96-well plates was 206 mM, approximately twice the concentration obtained in 24-well plates.

The effect of acetate tested in RKO cells (Figure 4.3-B) presented a similar pattern to HCT-15 cell line, in both cell culture plates used, and higher concentrations of acetate were also needed in 96-well plates. On both type of plates the percentages of cellular viability decreased as the concentration of acetate increased, although this decrease seemed to reach lower viability more rapidly in 24-well plates. Observing RKO cell line, the first concentrations tested in 24 and 96-well plates, 50 mM and 110 mM respectively, resulted in a similar decrease of the percentage of cellular viability. Analysing the remaining concentrations of acetate tested in both plates, the decrease observed in the 24-well plate (corresponding to the concentrations of 70, 110, 140 and 220 mM) was more abrupt. The acetate IC_{50} value determined for 24-well plate was 74 mM.

Regarding 96-well plates the effect of the remaining concentrations (125, 140, 180, 220, 300 and 400 mM) resulted in a more gradual decrease in the viability of RKO cells. According to the responses obtained for the RKO cell line with the 96-well plates 140 mM of acetate was estimated as the IC_{50} value.

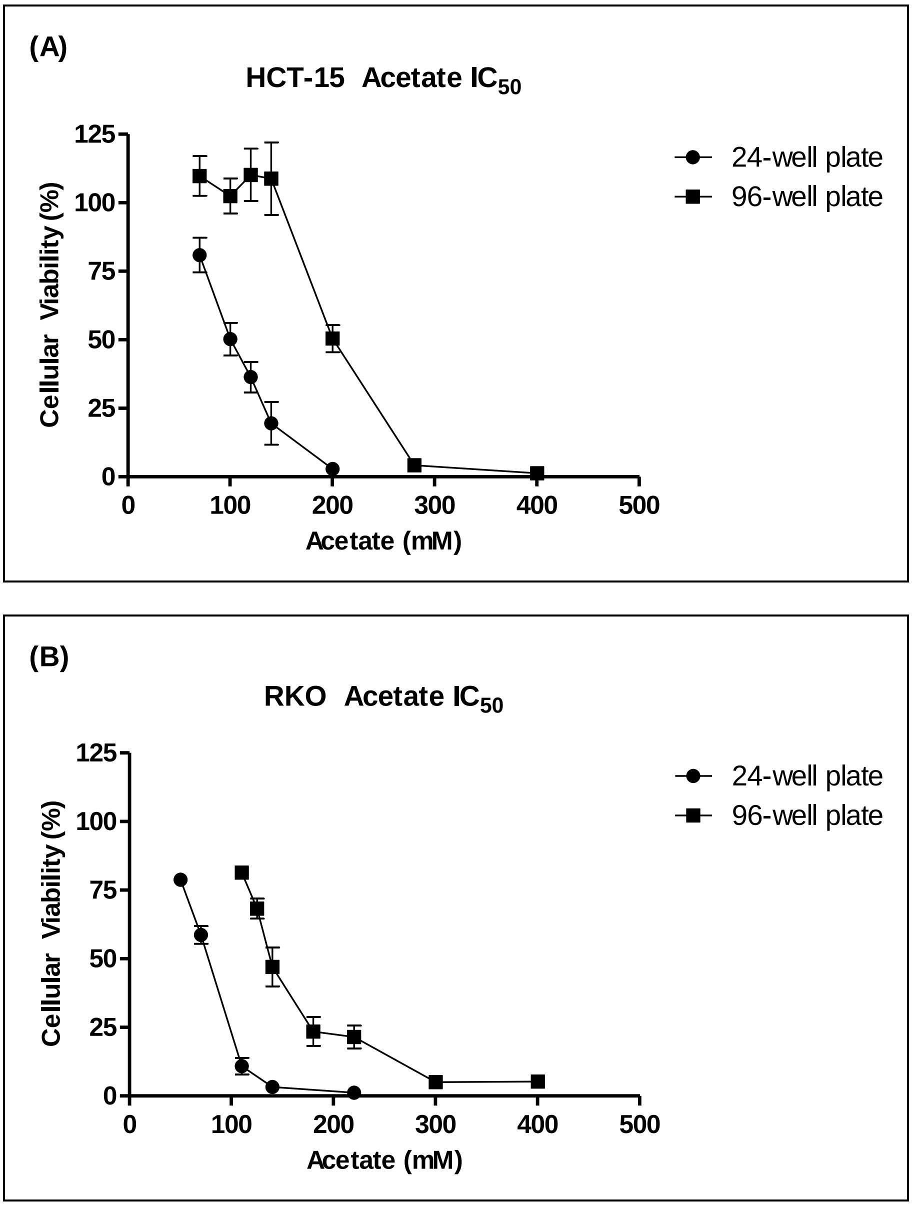


Figure 4.3 – Effect of different concentrations of acetate on cellular viability of HCT-15 **(A)** and RKO **(B)** cell lines, determined by MTT assay. HCT-15 and RKO cellular density was $7x10^4$ and $2,5x10^4$ cells/well, in 24-well plates, and $1x10^4$ and $7,5x10^3$ cells/well, in 96-well plates, respectively. Acetate (3M) treatment latest 48h before cells were analysed by MTT assay to determine the cellular viability. The percentage of viability was calculated relatively to the value of absorbance obtained for the negative control (untreated cells).

4.3 Effect of ceramide pathway inhibition on acetate-induced cell death in CRC cells

The next step in the project was to evaluate the effect of different inhibitors of specific steps of ceramide metabolism, alone or in combination, in the response to acetate in CRC cells. It was decided to test both the effect of pre-incubation with the inhibitor followed by the treatment with acetate alone, and the effect of the inhibitor along with treatment with acetate (co-incubation), on the loss of cell viability induced by acetate. We used C2-ceramide as a positive control in order to show that these cells were responsive to increased levels of ceramide as already previously demonstrated by our group (Silva, 2012 ; Master thesis).

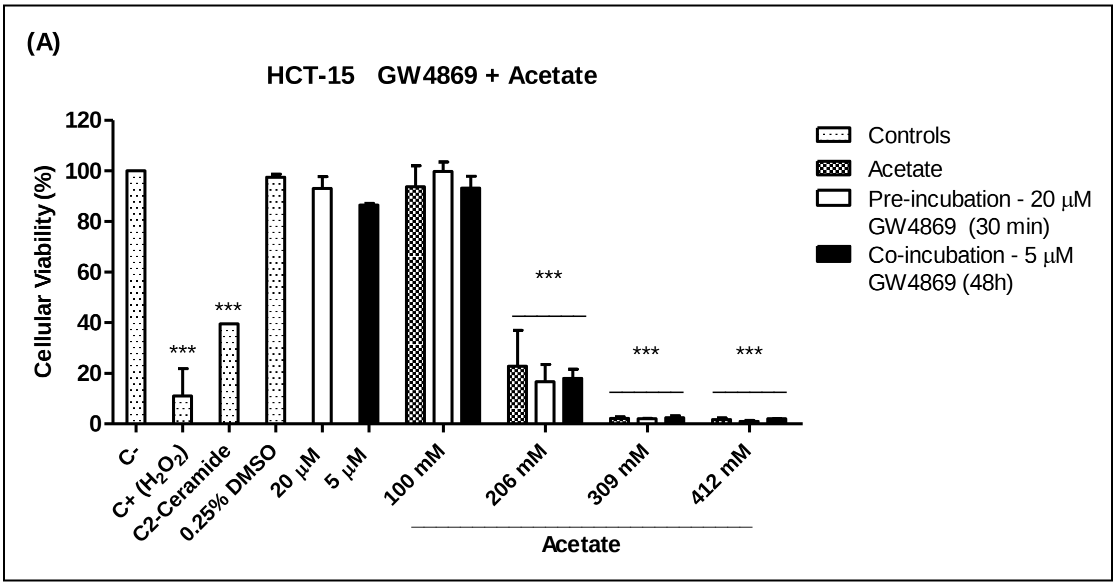
4.3.1 Effect of GW4869 and fumonisin B_1 in the acetate response on CRC cell lines

The effect of GW4869 or FB_1 in combination with acetate was evaluated. From now on all the assays were performed in 96-well plates, in order to save reagents and material in the experimental work. The acetate concentrations used are presented in Table II (in some cases, other concentrations different than those listed on Table II, were used to obtain additional information).

Table II – Concentrations of acetate used in 96-well cell culture plates for MTT assay.

	[Acetate] (mM)	
	HCT -15	RKO
IC_{50}	206	140
intermediate IC_{50}	309	210
2 x IC_{50}	412	280

The cell lines studied were treated with 20 µM of GW4869, during 30 minutes (pre-incubation treatment) or with 5 µM of GW4869, during 48h (co-incubation treatment). For the fumonisin B_1 assays, 60 µM were used in pre-incubation conditions and 10 µM in co-incubation conditions. In all the conditions tested, acetate was added during 48h and, in the case of co-incubation, acetate was added to the cells at the same time as the inhibitor.



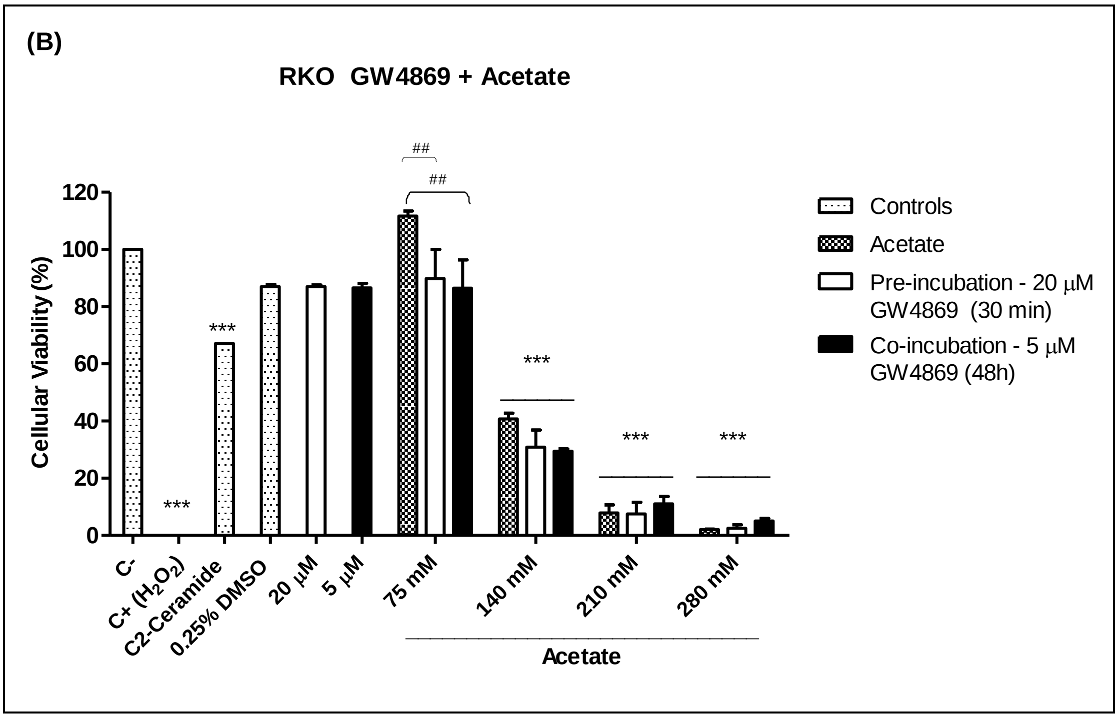


Figure 4.4 – Incubation of HCT-15 **(A)** and RKO **(B)** cell lines with GW4869 and acetate and determination of cellular viability, using MTT assay. HCT-15 and RKO were seeded, in 96-well plates, at a density of $1x10^4$ and $7,5x10^3$ cells/well, respectively. Pre-incubation represents treatment with 20 μM of GW4869, followed by 48h of treatment with acetate. Co-incubation represents treatment with 5 μM of GW4869 and acetate for 48h. Negative control (C-) corresponds to cells incubated with fresh complete medium and the positive control (C+) corresponds to cells treated with 500 μM (A) and 1,5 mM (B) of hydrogen peroxide (H_2O_2) and C2-ceramide (35 μM). The percentage of viability was calculated relatively to the value of absorbance obtained for the negative control. For each bar, mean ± SEM for two independent experiments (n=2) is represented. * p≤0.05; ** 0.01≤p≤0.001; *** p≤0.0001, compared to control cells (C-); ## 0.01≤p≤0.001 for comparison between treatments.

Cellular viability assays of both cell lines with the inhibitor of the nSMase (GW4869) and acetate, using pre- and co-incubation conditions, showed that the cellular viability values were not significantly different, i.e. the inhibitor did not change significantly the effect of acetate on cellular viability. In fact, when HCT-15 cells were submitted to pre- or co-incubation conditions either small increases or decreases in cellular viability could be observed compared to the control with acetate, but none of these alterations were enough marked to really sustain a (protective or synergistic) action of GW4869 upon acetate-induced apoptosis (Figure 4.4-A). Concerning the RKO cell line equivalent conclusions could be drawn, though a slight increase in cellular viability of RKO cell line for co-incubation with acetate 210 and 280 mM was observed, the difference was not of statistical significance (Figure 4.4-B).

The effect of fumonisin B_1, inhibitor of the CerS, on acetate induced loss of cellular viability of both cell lines was also assessed under pre- and co-incubation conditions (Figure 4.5).

The results obtained with HCT-15 cells demonstrated that, like GW4869, FB_1 caused slight alterations in the loss of cellular viability induced by acetate both under pre- or co-incubation conditions with the inhibitor. Nevertheless, for the IC_{50} of acetate (206 mM) there was a significant difference between the incubation with acetate alone and the pre- and co-incubation (treatment with acetate and FB_1). This decrease on the cellular viability was not expected assuming that ceramide mediates acetate-induced cell death. According to this hypothesis inhibition of ceramide metabolism would lead to an increase in cellular viability. However, this sensitizing effect of FB_1 was no more detected for treatment with the acetate intermediate IC_{50} (309 mM) and the 2 x IC_{50} (412 mM) values.

Concerning the RKO cell line, a similar behaviour was observed. Indeed a significant decrease was observed when cells were treated with 140 mM of acetate (IC_{50}) and co-incubated with FB_1, compared with cells treated with 140 mM of acetate. This decrease was also detected for 2 x IC_{50} of acetate, under co-incubation conditions but was not statistically significant.

Taking into account the results obtained it was decided to perform only co-incubation assays with the ceramide inhibitors since there was no relevant differences between the results obtained for the pre and co-incubation assays.

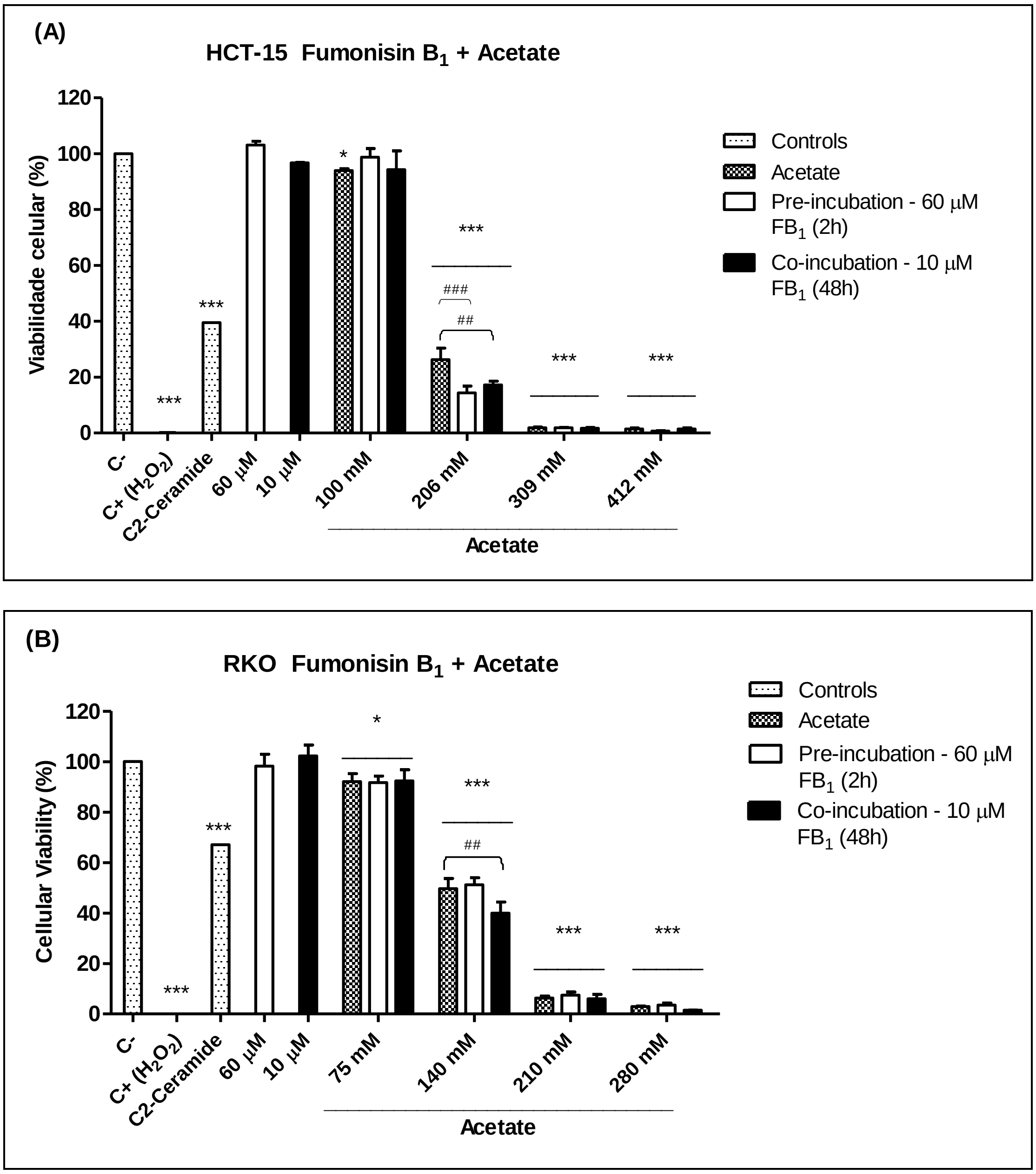


Figure 4.5 – Incubation of HCT-15 **(A)** and RKO **(B)** cell lines with fumonisin B_1 and acetate and determination of cellular viability, using MTT assay. HCT-15 and RKO were seeded, in 96-well plates, at a density of $1x10^4$ and $7,5x10^3$ cells/well, respectively. Pre-incubation represents treatment with 60 µM of FB_1, followed by 48h of treatment with acetate. Co-incubation represents treatment with 10 µM of FB_1 and acetate for 48h. Negative control (C-) corresponds to cells incubated with fresh complete medium and the positive control (C+) corresponds to cells treated with 500 µM (A) and 1,5 mM (B) of hydrogen peroxide (H_2O_2) and C2-ceramide (35 µM). The percentage of viability was calculated relatively to the value of absorbance obtained for the negative control. For each bar, mean ± SEM for three independent experiments (n=3) is represented. * p≤0.05; ** 0.01≤p≤0.001; *** p≤0.0001, compared to control cells (C-); ## 0.01≤p≤0.001, ### p≤0.0001 for comparison between treatments.

4.3.2 Effect of GW4869, fumonisin B_1 and myriocin in acetate-induced cell death in CRC cells

To further explore the inhibition of ceramide biosynthesis pathways and to elucidate if acetate really triggers apoptosis via ceramide, it would be necessary to block all the major pathways that lead to ceramide generation. Therefore including another inhibitor of the ceramide pathways in the experiments seemed to be relevant and these results were previously obtained by our research team (Silva, 2012 ; Master thesis).

Myriocin, a compound that inhibits the action of SPT (serine palmitoyltransferase) was included in the experiments to assess the role of the *de novo* synthesis pathway blockage. The concentration of this inhibitor to be used had already been established in a previous work of our group and was 150 nM for the two cell lines (Silva, 2012 ; Master thesis). The use of myriocin alone or together with GW4869 (5 μM) and FB_1 (10 μM) could indicate, in case it causes a decrease in the loss of cellular viability induced by acetate, that ceramide is involved in acetate induced cell death.

Results of the experiments with HCT-15 and RKO cell lines were similar. For the HCT-15 cell line the combined effect of GW4869, FB_1 and myriocin did not lead to an increase in cellular viability, comparing with cells treated only with acetate (Figure 4.6-A). Instead all the treatments induced a decrease on cellular viability similar to acetate and statistically significant, when compared with the negative control.

The data collected from HCT-15 cells treated with acetate and the three inhibitors of the ceramide biosynthesis pathways (Figure 4.6-A) demonstrated that there was no noteworthy alteration on cellular viability. This result was particularly noticed when cells were treated with the intermediate IC_{50} (309 mM) and the 2 x IC_{50} (412 mM) doses that led to a similar loss of cell viability. With the IC_{50} dose of acetate (206 mM), it was more evident that there were no important changes in viability between the different conditions tested. When cells were incubated with acetate and the three drugs (GW4869, FB_1, and myriocin), the percentage of viability was lower than cells treated only with acetate, cells treated with acetate and FB_1, and cells incubated with acetate and a combination of inhibitors (GW4869 + myriocin; FB_1 + myriocin). These alterations on the viability of HCT-15 cells were not statistically significant and were not sufficient to affirm that these changes were due to the inhibition of the ceramide pathways and to associate its inhibition to acetate-induced cell death. Moreover, when cells submitted to all the treatments tested were observed under the microscope (Figure 4.7), it remained difficult to notice any pertinent change on the cell number or phenotype.

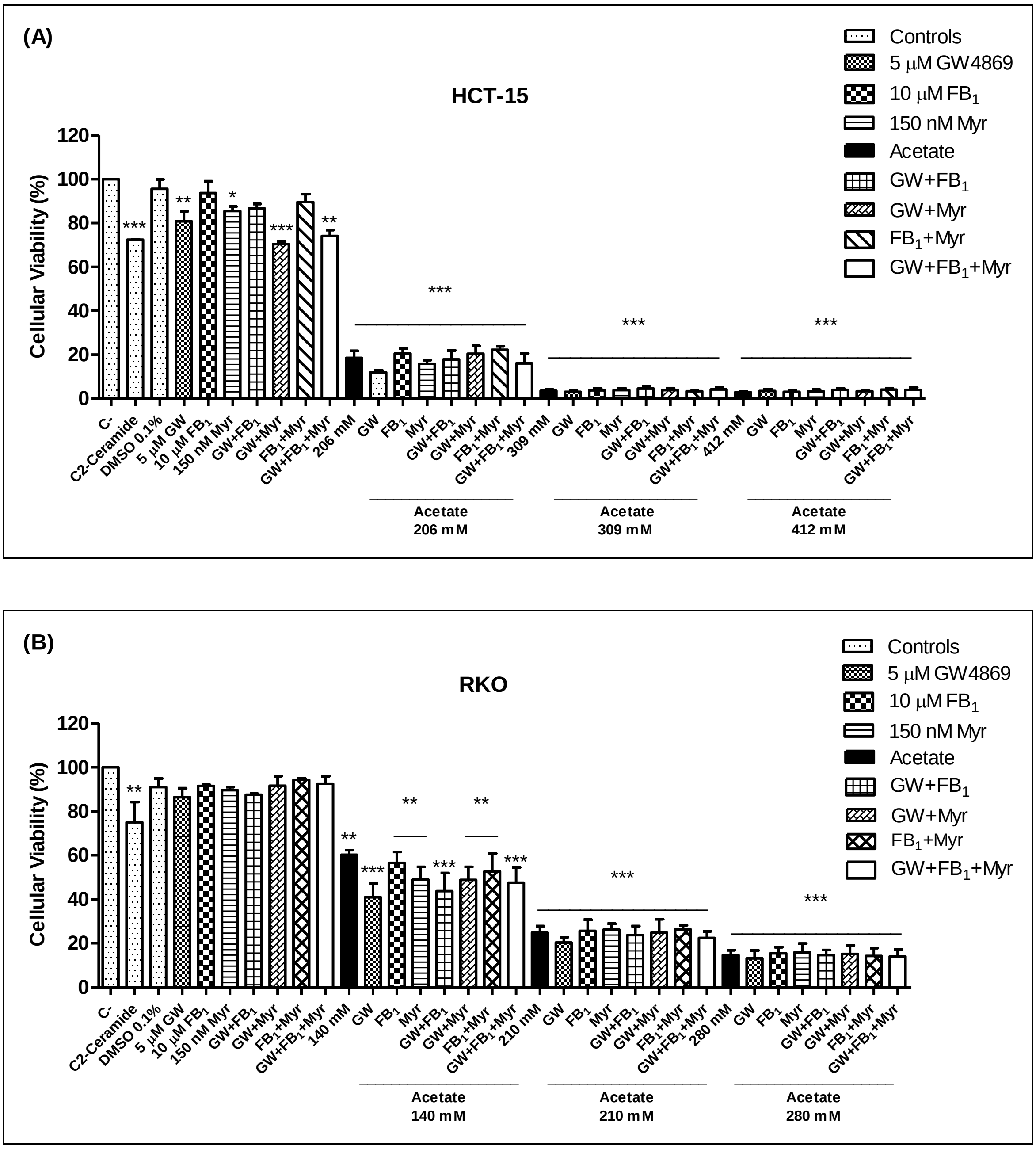


Figure 4.6 – Incubation of HCT-15 **(A)** and RKO **(B)** cell lines with GW4869, fumonisin B_1, myriocin and acetate and determination of cellular viability using MTT assay. HCT-15 and RKO were seeded, in 96-well plates, at a density of $1x10^4$ and $7,5x10^3$ cells/well, respectively. Cells were incubated with 5 µM of GW4869, 10 µM of FB_1, 150 nM of myriocin and acetate for 48h. Negative control (C-) corresponds to cells incubated with fresh complete medium and the positive control (C+) corresponds to cells treated C2-ceramide (35 µM). The percentage of viability was calculated relatively to the value of absorbance obtained for the negative control. For each bar, mean ± SEM for two independent experiments (n=2) is represented. * p≤0.05; ** 0.01≤p≤0.001; *** p≤0.0001, compared to control cells (C-).

The RKO cell line displayed the same type of outcome as observed in HCT-15 cell line (Figure 4.6-B). There were no statistical significant differences between the treatment with acetate alone and treatment with acetate and the inhibitors. When cells were treated with the IC_{50} dose (140 mM) of acetate, the incubation with acetate alone led to a loss of cell viability lower than that observed for all the other conditions.

For the incubation of RKO cells with the intermediate IC_{50} (210 mM) and the 2 x IC_{50} (280 mM) acetate doses and with the inhibitors alone or in combination, some treatments led to a no statistically significant increase in the percentage of cellular viability when compared with acetate. Additionally, the treatment with acetate and the three drugs (GW4869, fumonisin B_1 and myriocin) revealed no major alterations on cell viability. This result was also observed for cells treated with the inhibitors combined in different pairs (GW4869 + fumonisin B_1; GW4869 + Myriocin; fumonisin B_1 + Myriocin). Phase contrast photographs visualization (Figure 4.8), just before completing 48h of treatment, revealed subtle differences between treatments, which are in accordance with the histograms obtained.

Gathering and analysing all the data collected, we can conclude that no major alterations could be observed concerning cellular viability, on the attempt to revert the effect of acetate in inducing cell death, using the compounds (GW4869, fumonisin B_1 and myriocin) that interfere with the biosynthesis of ceramide and its signalling pathways. In summary, ceramide signalling does not seem to be relevant in the cell death induced by acetate in CRC cells.

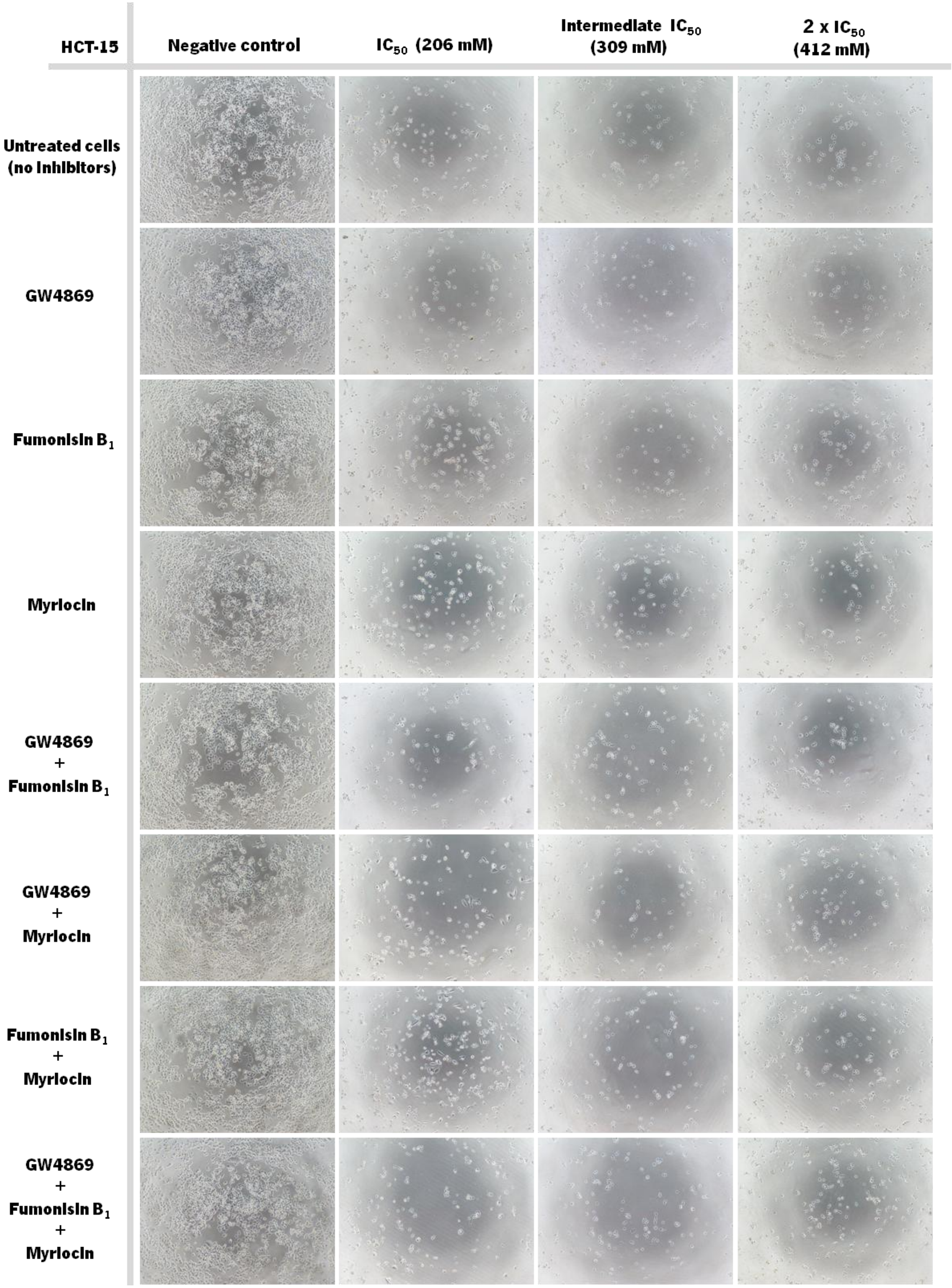


Figure 4.7 – HCT-15 cells, incubated (48h) with acetate and inhibitors of ceramide metabolism (GW4869, fumonisin B_1 and myriocin), observed under the microscope, on phase contrast (100x).

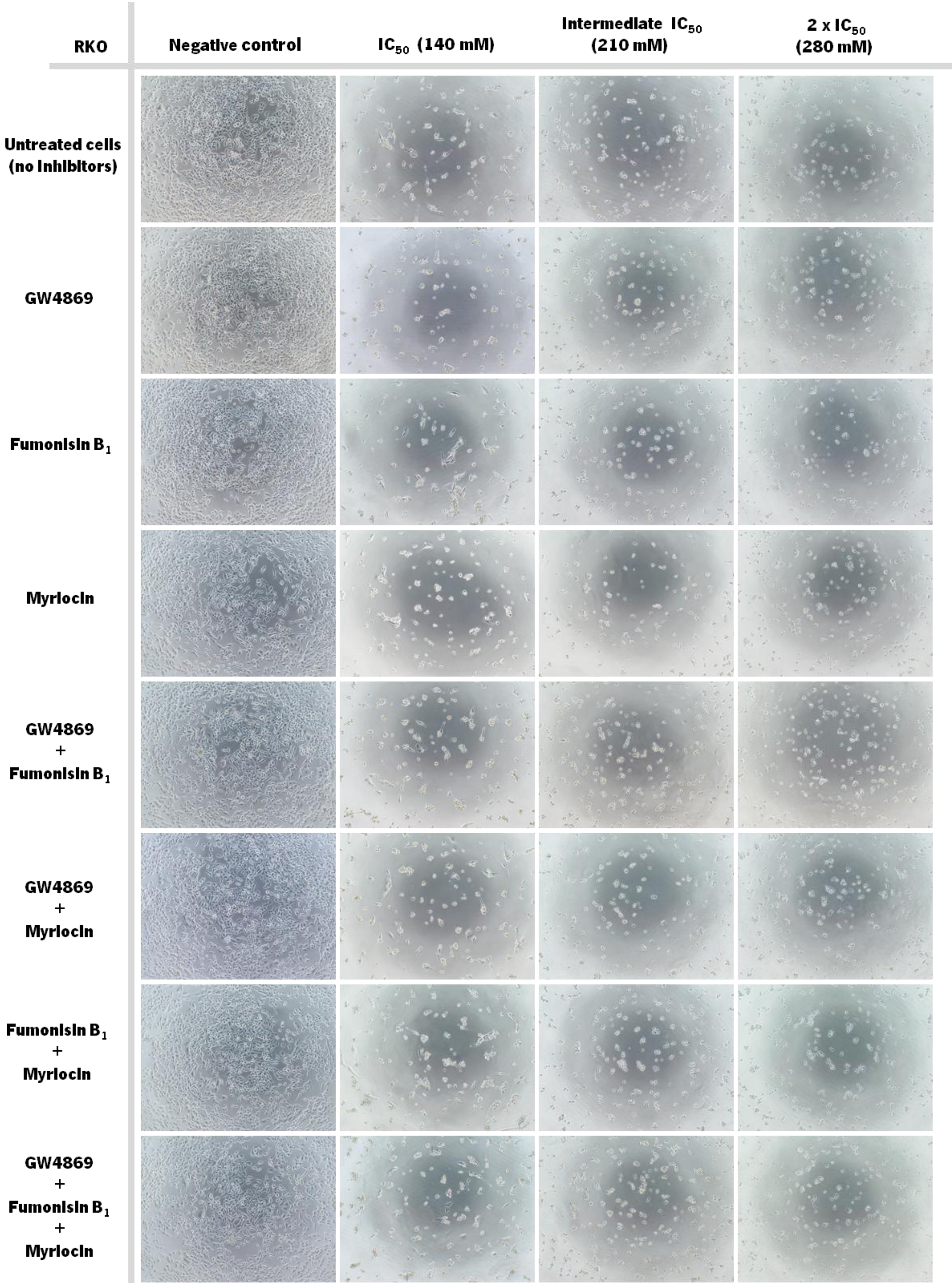


Figure 4.8 – RKO cells, incubated (48h) with acetate and inhibitors of ceramide metabolism (GW4869, fumonisin B_1 and myriocin) - 48h, observed under the microscope, on phase contrast (100x).

5

Discussion

5.1 Effect of ceramide pathways inhibitors on acetate-induced cell death

Over the years, in the attempt of finding new therapy strategies to use in the prevention and treatment of colorectal carcinoma (CRC), some studies reported that bacteria of the genus *Propionibacterium* had the capacity to kill human CRC cells, *in vitro*, triggering a mechanism known as apoptosis or programmed cell death (Jan, Belzacq *et al.*, 2002). A protective role played by these bacteria was also found to occur *in vivo* in mouse models (Lan, Lagadic-Gossmann *et al.*, 2007; Lan, Bruneau *et al.*, 2008). The protection resides not on the bacteria itself, but on short chain fatty acids (SCFAs), such as butyrate, propionate and acetate, which are products of the fermentative metabolism of these bacteria. These compounds were found to be, at least in part, responsible for killing CRC derived cells lines, namely HT-29 and Caco 2 cell lines (Jan, Belzacq *et al.*, 2002).

Results obtained in the present project confirm that acetate is able to induce cell death in two different CRC-derived cell lines HCT-15 and RKO, in accordance with previous findings from our group that acetate triggers apoptosis in those CRC-derived cell lines (Marques, Oliveira *et al.*, 2012 ; submitted). Various markers of apoptosis were found to take place after acetate treatment such as DNA fragmentation, phosphatidylserine exposure to the outer leaflet of the plasma membrane, caspase activation and the emergence of a sub-G1 cell population (Marques, Oliveira *et al.*, 2012 ; submitted).

The same observation had already been made by Jan research team, which stated that SCFAs (namely acetate and propionate) were responsible for inducing apoptotic features in colorectal carcinoma cells. Chromatin condensation, nuclei shrinkage and fragmentation, activation of caspase 3 and formation of apoptotic bodies were observed. In addition, evidences found implicated mitochondria in the process including increased mitochondrial inner membrane permeability, ROS accumulation, and involvement of ANT, a putative SCFA target, in the cell death mechanism (Jan, Belzacq *et al.*, 2002).

In *S. cerevisiae*, acetic acid has proved to induce mitochondria-mediated apoptotic process displaying similar characteristics as found in SCFAs-mediated cell death in CRC cells. Indeed, ACC carriers (the mitochondrial yeast orthologues of the mammalian adenine nucleotide translocator, ANT) are required for mitochondrial outer membrane permeabilization (MOMP), cytochrome *c* release in yeast apoptosis (Pereira, Camougrand *et al.*, 2007) and ANT, a putative component of the mammalian PTPC (permeability transition pore complex), was suggested as a potential SCFA target (Jan, Belzacq *et al.*, 2002). Moreover, it was found that in both cases the mitochondrial dysfunctions induced by SCFAs, in CRC cells, can also be partially inhibited by expression of anti-apoptotic members of the Bcl-2 protein family (Jan, Belzacq *et al.*, 2002; Saraiva, Silva *et al.*, 2006).

Recently it was found that Pep4p (the yeast orthologue of human cathepsin D) is released from the vacuole to the cytosol, in cells undergoing acetic acid-induced apoptosis, and it plays a protective role in the active cell death process (Pereira, Chaves *et al.*, 2010). Notably, in accordance with this observation our group showed that acetate also triggers LMP (lysosome membrane permeabilization) and cathepsin D release to the cytosol in HCT-15 and RKO CRC derived cell lines. Moreover, we found that inhibitors of cathepsins (including cathepsin D) enhanced acetate-induced apoptosis of CRC cells, suggesting a potential protective role of cathepsin D against apoptosis in CRC cells (Marques, Oliveira *et al.*, 2012 ; submitted) similar to Pep4p. Indeed deletion of Pep4p conferred a higher susceptibility of yeast to acetic acid-induced apoptosis (Pereira, Chaves *et al.*, 2010).

Besides being not well understood, the relation of cathepsin D with CRC have revealed that its expression might be higher in tumour cells (Yilmaz, Uzunlar *et al.*, 2003) as also proved in HCT-15 and RKO cells (Marques, Oliveira *et al.*, 2012 ; submitted). Therefore, expression of cathepsin D in CRC might be beneficial to its progression and this molecule could be a potential target in CRC therapy.

The results of the present study showed that acetate leads to a significant reduction in cellular viability of HCT-15 and RKO CRC derived cell lines, which is in accordance with our group and others groups observations.

On both cell lines studied, the acetate IC_{50} determinated in 96-well plates was twice the IC_{50} determinated in 24-well plates (Table I and Figure 4.3). This fact might be explained by the difference between the size of the well, the cellular distribution and interactions between the cells and the culture environment. The 24-wells have higher dimensions, thus the cells are more distant from each other, despite a higher number of cells being seeded on these plates and, therefore, providing a higher contact between acetate and cells. The interaction of the cells with their environment, like gas exchange (O_2 and CO_2) for example, might influence the response to acetate.

The balance between proliferation and cellular death is a key aspect in tumour development and biological active sphingolipids play a major role in the regulation of various processes that are involved in cancer pathogenesis (Ogretmen and Hannun, 2004). Ceramide is one of these important lipids which are indispensable in the regulation of mechanisms such as cell growth, senescence, cell death, adhesion, migration, inflammation, angiogenesis, and intracellular trafficking (Hannun and Obeid, 2008). As a consequence, the major objective of the current project was to understand if ceramide is involved in the response to acetate on colorectal cancer cell lines. In the attempt of elucidate this particular topic, we aimed to test whether interfering with ceramide biosynthetic pathways affects acetate induced cell death. A support to this idea is that several chemotherapeutic or chemopreventive agents were reported to lead to cell death (mostly through apoptosis), associated with an increase of ceramide endogenous levels (Moussavi, Assi *et al.*, 2006; Huang, Chen *et al.*, 2011). Also, a study of human colon cancer revealed that the levels of ceramide are 50% lower than those found in normal colon mucosa (Selzner, Bielawska *et al.*, 2001), and that application of ceramide analogues and ceramidase inhibitors, activates various pro-apoptotic molecules. Thus, it seems to be important to look whether the intracellular pool of ceramide is altered during acetate-induced cell death and the effect of inhibitors ceramide metabolism on cell survival.

Three principal inhibitors of the ceramide pathways were used: GW4869, fumonisin B_1 and myriocin. GW4869 is a compound that inhibits the neutral sphingomyelinase (nSMase) that is part of the SMase pathway, both *in vitro* and *in vivo* (Luberto, Hassler *et al.*, 2002); fumonisin B_1 is known to act on the salvage and *de novo* synthesis pathways of ceramide, inhibiting both the acylation of sphingosine and dihydrosphingosine by ceramide/dehydroceramide synthase (CerS/LASS) (Kitatani, Idkowiak-Baldys *et al.*, 2008); and myriocin is a serine palmitoyltransferase (SPT) inhibitor acting on the *de novo* synthesis pathway.

5.1.1 Inhibition of the sphingomyelinase pathway: effect of GW4869 on cellular viability of CRC cell lines

The experiments with GW4869 and acetate (Figure 4.4 and 4.6) did not show an increase in cell viability when cells were co-treated with these two compounds. Our results showed that inhibition of the SMase pathway did not revert, in a significant manner, the loss of viability caused by acetate alone, on both cells lines studied. Neither the pre-incubation or co-incubation condition made a difference on the results observed (Figure 4.4). Such observation could be a consequence of the existence of alternative pathways, for ceramide synthesis, that would compensate this inhibition. It is also known that three mammalian forms of nSMases exists (nSMases 1-3) and they play important roles in signalling processes (Figure 5.1). Several cellular stresses, represented in Figure 5.1, have been shown to activate nSMases, including various members of the tumour necrosis factor (TNF) receptor family, and anticancer drugs (Chatterjee, 1999; Krönke, 1999; Levade and Jaffrézou, 1999).

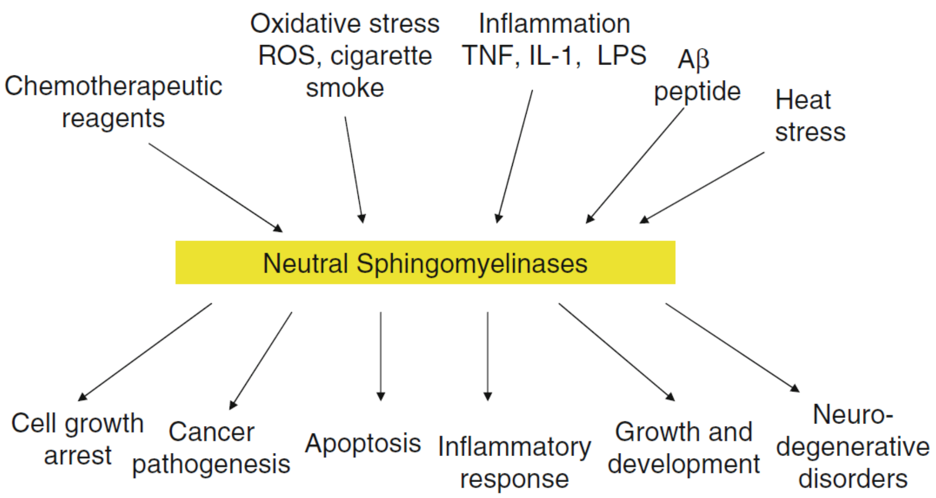


Figure 5.1 – Signalling roles of neutral sphingomyelinases in response to potential activators. Adapted from (Wu, Clarke *et al.*, 2010).

The nSMase 2 is located in the plasma membrane of cells, and GW4869 was recognized as a nSMase 2 inhibitor, both *in vitro* and *in vivo* (Wu, Clarke *et al.*, 2010). This enzyme seems to be implicated in cell growth as a study employing MCF7 (breast cancer) cell line, overexpressing nSMase2 in cells, showed a 40% diminution in the sphingomyelin levels and accelerated sphingomyelin catabolism, leading to an increase of 60±15 % of ceramide levels in nSMase2-overexpressing cells (Marchesini, Luberto *et al.*, 2003). The stable overexpression of nSMase2 led to a (30-40%) decrease in the growth rate at the late exponential phase.

A study by Luberto and colleagues, in 2002, demonstrated that GW4869 protected, in a dose-dependent manner, MCF7 cells from TNF-induced apoptosis, acting also in the inhibition of cytochrome *c* release from mitochondria and caspase 9 activation (Luberto, Hassler *et al.*, 2002). In this study, cells were pre-treated for 30 min with either 10 or 20 μM of GW4869, followed by a TNF treatment for 24h. Maybe if we had tested, in the present project, different time points besides 48h, we could have obtained different results, exhibiting the protective effect verified by Luberto.

Furthermore, GW4869 inhibitor failed to block the novel histone deacetylase inhibitor LAQ824-mediated apoptosis in leukaemia cells (U937) (Rosato, Maggio *et al.*, 2006). These results suggest that acetate-induced cell death might not be directly linked to ceramide synthesis by nSMase, at least in HCT-15 and RKO cell lines, as observed in the present study. Determination of nSMase activity before and after treatment with acetate would allow supporting this interpretation.

5.1.2 Inhibition of de salvage and *de novo* synthesis pathways: effect of fumonisin B_1 on cellular viability of CRC cell lines

The effect of fumonisin B_1, an inhibitor of the ceramide synthase (CerS) that acts on the salvage and *de novo* pathways of ceramide, on acetate-induced apoptosis in HCT-15 and RKO cell lines, was also tested. As seen on Figure 4.5 and 4.6, the expected increase in the percentage of viable cells when treated with the inhibitor and acetate, did not occur. Rather a significant decrease in cell viability was observed in HCT-15 and RKO cell lines after pre- and co-incubation with 206mM of acetate and after co-incubation with 140 mM of acetate, respectively (Figure 4.5).

Fumonisins have been extensively employed to understand the function of ceramides and other sphingolipids in cultures of mammalian cell lines. Somewhat in a paradoxal way, a certain number of studies use fumonisins to induce apoptosis and, in other contexts, these compounds are growth stimulatory and anti-apoptotic rather than cytotoxic (Desai, Sullards *et al.*, 2002).

Studies have previously demonstrated that, in anticancer drug mediated apoptosis scenarios, such as of daunorubicin-induced apoptosis in leukaemia cells - P388 and U937 cell lines, FB_1 was able to block the elevation of ceramide levels and apoptosis (Bose, Verheij *et al.*, 1995). In colorectal cancer models, previous experiments showed that when FB_1 (10 μM) was used with a chemotherapeutic agent – Irinotecan – CRC cell death diminished significantly and increased in 95% cell viability (Litvak, Bilchik *et al.*, 2003). The increase in sphingosine-1-phosphate is likely to be a considering factor in cells where fumonisins are growth stimulatory instead of toxic and could play a major role in apoptosis inhibition. On the other hand, when FB_1 is added to cells in culture, it causes elevations in sphinganine (due to the inhibition of *de novo* pathway), and results most of the times in growth arrest (Ciacci-Zanella, Merrill Jr *et al.*, 1998) and apoptosis (Schmelz, Dombrink-Kurtzman *et al.*, 1998). This could be one of the reasons behind the decrease in the percentage of viability in HCT-15 and RKO cells submitted to FB_1 and acetate treatment.

The inhibition by FB_1 may result in the increase of free sphingoid bases and their 1-phosphates and decreased ceramide biosynthesis. The inhibition of ceramide synthase can inhibit cell death (ceramide-induced), but promote free sphingoid base-induced cell death. It is the balance between the change in anti- and pro-apoptotic sphingolipid species (e.g. decreased ceramide and increased sphingosine-1-phosphate levels or increased ceramide, free sphingoid bases and altered fatty acids levels) that will determine the cellular response - Figure 5.2 (Riley, Enongene *et al.*, 2001). Hypothetically in the present study, the inhibition by FB_1 may cause an elevation of sphingosine levels, which has pro-apoptotic effect, as represented in the sphingolipid rheostat, thus justifying the fact that there is a decrease in cell survival in both cell lines.

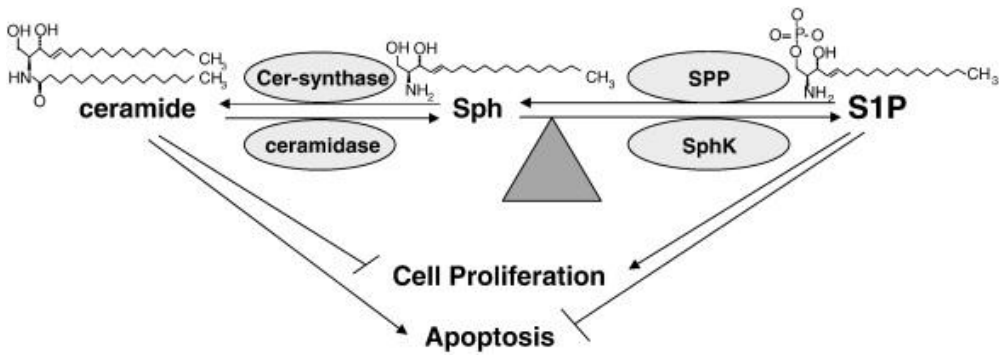


Figure 5.2 – Schematic representation of the sphingolipid rheostat. Ceramide (and sphingosine) favour a block in cell proliferation and apoptosis while sphingosine-1-phosphate (S1P) favours proliferation and cell survival. Thus sphingosine kinase (SphK) is in an important position to favour cell survival. SPP, sphingosine-1-phosphate phosphatase; Cer-synthase, ceramide synthase. Adapted from (Van Brocklyn and Williams, 2012).

It is also important to refer that HCT-15 and RKO cells have different sphingosine kinase (SphK) activities. RKO has a much higher activity of both SphK1 (57.90 ± 11.5 pmol/min/mg protein) and SphK2 (68.81 ± 11.05 pmol/min/mg protein) than HCT-15 (8.38 ± 1.05 and 4.82 ± 1.34 pmol/min/mg protein, respectively) (Nemoto, Nakamura *et al.*, 2009). Although SphK1 and SphK2 are closely related, use the same substrate, sphingosine, and generate the same product, S1P, they seem to have contrary roles in cells. While SphK1 has a pro-survival role, SphK2 is associated with cell growth inhibition and apoptosis (Maceyka, Sankala *et al.*, 2005). A probable explanation to these contrasting functions, given by Maceyka and collaborators, is the fact that the two proteins are located or translocated to different parts of the cell, and that the localized production of S1P has distinct functions; also SphK2 might act in concert with S1P phosphatase to convert S1P into sphingosine and then ceramide.

These differences in enzyme activity, between the two cell lines, may explain the different behaviours observed justifying, for instance, HCT-15 being more prone to survival. Generally, when treated with acetate, HCT-15 is more resistant to acetate than RKO, because higher doses of acetate are necessary to diminish the cellular viability of HCT-15 cells in 50%. The higher activity of SphK1/2 found in RKO cells might play a decisive role in the response to this inhibitor, explaining in part the results obtained. Furthermore, the levels of SphK2 (pro-apoptotic) are slightly higher than SphK1 (pro-survival) in RKO, in opposition to HCT-15, which may explain why a higher dose of acetate is needed to induce cell death in HCT-15.

5.1.3 Inhibition of the *de novo* synthesis pathway: effect of myriocin on cellular viability of CRC cell lines

Myriocin, as described before is an SPT inhibitor acting on the generation of ceramide by the *de novo* pathway. This compound inhibits both mammal and fungal SPT in cell free preparations and its IC_{50} values are included in the nanomolar (nM) range (Miyake, Kozutsumi *et al.*, 1995; Hanada, 2003).

From the results obtained by our research group (Silva, 2012 ; Master thesis) and in order to observe the influence of the *de novo* synthesis pathway in acetate-induced cell death in HCT-15 and RKO cell lines, 150 nM of myriocin were used in each experiment (Figure 4.6) in co-incubation with acetate, for 48h. As observed, there are no meaningful changes in the cellular viability of both HCT-15 and RKO cell lines after the treatment of acetate in combination with this inhibitor, revealing that this pathway may not be directly involved in acetate signalling cell death. These results are in accordance with the ones previously obtained by our research team (Silva, 2012 ; Master thesis).

The hindrance of the *de novo sphingolipid* pathway, alone, did not reverse the effect of acetate on the cell lines studied. The generation of ceramide through other pathways may be considered, as they could compensate the action of myriocin. Nonetheless, when using this compound, we have to have in mind the fact that it is reported that when used for several hours it may impede all sphingolipid synthesis and, also, the synthesis of complex sphingolipids (Kitatani, Idkowiak-Baldys *et al.*, 2008).

On the other hand, myriocin has been found to induce growth inhibition, in melanoma cells (Lee, Choi *et al.*, 2001), reducing the levels of ceramide, sphingomyelin, sphingosine and sphingosine-1-phosphate. Myriocin has also been shown to play a central role in ceramide levels regulation and partially reverting curcumin's apoptosis-inducing death in colon cancer cell lines (Moussavi, Assi *et al.*, 2006). In this study, Moussavi and collaborators reported that the induction of apoptosis by curcumin is accompanied with intense ceramide generation, which could be attenuated by pre-incubation with myriocin and, also, that cell death could in part be reversed by this inhibitor. Therefore, it was important to observe the effect of myriocin in the conditions used in the present study, because acetate-induced cell death could lead to a comparable outcome (reversion of the viability loss, in the present case) if the *de novo* ceramide synthesis is signalling the process of cell death.

5.1.4 Effect of inhibiting different ceramide pathways on the cellular viability of HCT-15 and RKO cell lines

To further explore the role of ceramide biosynthesis involvement in acetate-induced cell death, in CRC cell lines, different inhibitors of ceramide pathways were used simultaneously, in different combinations (Figure 4.6).

To begin with, GW4869 and fumonisin B_1 were incubated together with acetate, as those two compounds would allow blocking the principal pathways of ceramide synthesis i.e. the SMase pathway and the salvage and the *de novo* pathways. As seen in Figure 4.6, the conjugated incubation tested did not alter in a significant manner the percentages of viability, neither in HCT-15 nor in RKO cells, comparing to the acetate effect alone. These inhibitors were also incubated separately with myriocin, thus inhibiting simultaneously the *de novo* pathway and the SMase pathway (when incubated with GW4869) or inhibiting the *de novo* pathway at two different stages (when incubated with fumonisin B_1). These different combinations of inhibitors did not result in a significant alteration of the cellular viability on both cell lines - Figure 4.6, though a slight increase (not statistically different) in cellular viability was observed when HCT-15 cells were treated with 206 mM of acetate.

Having this result in mind, a last combination of inhibitors with acetate was tried: GW4869, FB_1 and myriocin were incubated all together with acetate, during 48h. With this last combination all the pathways above are inhibited and the blockage of the *de novo* pathway is reinforced. Nevertheless, the outcome was the same, although there was a tendency to induce an increase in the values of viability, it was not statistically significant, as it would be expected if ceramide signalling was relevant in acetate-induced cell death. Consequently, the sphingolipid metabolism and ceramide signalling do not appear to be involved in acetate-induced cell death.

Small alterations on the level of some sphingolipid species would trigger different cell responses as already described. Recently, it has been found that sphingolipid metabolism is important in mitochondria-mediated yeast apoptosis induced by acetic acid. The deletion of *ISC1*, orthologue of mammalian nSMase2, or of *LAG1*, orthologue of mammalian CerS/LASS1 (longevity assurance gene) improved the survival of yeast cells exposed to acetic acid (Rego, Costa *et al.*, 2012 ; accepted). Very often, the processes that occur in the yeast model are very similar to those happening in mammalian cells. Therefore, it would be fundamental to carry some additional experiments (the quantification of ceramide levels or tracing the ceramide metabolism, for instance) as approached in the next chapter, to fully exclude the involvement of ceramide in cell death mediated by acetate.

The effect of desipramine, another inhibitor of the sphingolipid metabolism, which was tested on HCT-15 and RKO cell lines in a previous work developed by our group, could also have been used. This compound is a tricyclic anti-depressant, which is known to specifically inhibit the acid sphingomyelinase (aSMase) in the SMase pathway of ceramide production (Kölzer, Werth *et al.*, 2004) and the acid ceramidase (Elojeimy, Holman *et al.*, 2006). The reason why we opted not to use it again on the present project is because of the toxicity demonstrated in which the acetate effect was accentuated (Silva, 2012 ; Master thesis). These findings were in accordance with what had been described about its cytotoxicity and its role in CRC apoptosis (Arimochi and Morita, 2006; Arimochi and Morita, 2008). On the other hand, this inhibitor might be very interesting as a potential CRC therapy drug, since its action increases the toxicity of certain chemotherapeutic drugs (Kabolizadeh, Engelmann *et al.*, 2012), and because it targets ceramide metabolism which is frequently deregulated in cancer (Ryland, Fox *et al.*, 2011).

The alteration of certain parameters in the experiments tested throughout the present project could perhaps result in different outcomes. For example, testing different times points such as 16, 24 and 32h, instead of only 48h, where cell survival might be too reduced to allow the observation of the effect of the different inhibitors tested. Furthermore, it would be useful to determine if the inhibitors used changed the intracellular ceramide levels, since it would permit the verification of their impact in sphingolipid metabolism on colorectal carcinoma derived cell lines.

Numerous studies demonstrated that increasing the cellular content of ceramide would lead to programmed cell death; others have shown that lower cellular ceramide levels interfere with apoptosis induced by other agents (Kolesnick and Kronke, 1998; Birbes, El Bawab *et al.*, 2002; Siskind, 2005). Moreover, previous studies performed by our research team indicate that treatment of HCT-15 and RKO cell lines with extrinsic ceramide (C2-ceramide) induced apoptosis on both cell lines, though RKO was more susceptible (Silva, 2012 ; Master thesis). These evidences are the consequence of two facets of ceramide: its metabolic conversion into sphingosine-1-phosphate or glucosphingolipids, which lead to cellular proliferation, or ceramide by itself that generates apoptosis (Huwiler and Zangemeister-Wittke, 2007). The balance between proliferation and cell death might be absent in cancer cells and if this balance (cellular homeostasis) is successfully restored it could constitute a putative therapy target against tumour development (Huwiler and Zangemeister-Wittke, 2007; Huang, Chen *et al.*, 2011).

It would be necessary to perform more experiments, for instance, to measure the quantities of ceramide present in the cells after the several treatments with inhibitors and acetate alone or in combination, in order to draw more reliable conclusions. It is important to have in mind that the distinct metabolic capacities of the cell lines (HCT-15 and RKO) and the exposure to different compounds, such as GW4869, FB_1 and myriocin (interfering in the ceramide production) could lead to distinct outcomes. Though, both cells lines ultimately behaved in the same way, the fact they have different genetic backgrounds and the intervention with several compounds in such a complex sphingolipid metabolism could have end up with different results. These facts could partially explain some of apparently contradictory results found in the literature, where in some cases using inhibitors of ceramide pathways reverse the effect of a given compound that induces cell death or, as it is presented in this project, no reversion is observed.

6

Conclusion and Future Perspectives

6.1 Final conclusion

The major goal of the project was to investigate the role of ceramide in acetate-induced apoptosis. In order to accomplish this objective we used two colorectal carcinoma cell lines, and treated them with three inhibitors of ceramide pathways: GW4869 (inhibitor of the sphingomyelinase pathway), fumonisin B_1 (inhibitor of the *de novo* and salvage pathways) and myriocin (inhibitor of the *de novo* pathway), to interfere with the ceramide biosynthesis.

Inhibitors were tested alone or in combination and in conjugation with acetate. The results achieved in the present work indicate that ceramide does not mediate acetate-induced cell death in HCT-15 and RKO cell lines. Different inhibitors of sphingolipid metabolism used alone or in combination did not reverse the effect of acetate on the decrease of cellular viability of both cell lines. In other words, the expected increase of viable cells with the inhibition of ceramide pathways was not observed. Therefore, our results suggest that none of the major pathways responsible for ceramide production namely the *de novo* pathway, the salvage pathway and the SMase pathway, seems to interfere in the acetate-induced cell death in CRC cells.

6.2 Future perspectives

Further exploitation of these results would be important to solidify the knowledge acquired within this project. The next step could be the evaluation of ceramide levels in the CRC cell lines tested before and after the different treatments, as it would give us an idea if changes in ceramide amount occur when incubated with acetate alone or acetate and inhibitors of ceramide biosynthesis pathways. An example of a technique that could be used to quantify the levels of ceramide could be the use of an anti-ceramide antibody (Grassmé, Jekle *et al.*, 2001; Cianchi, Cortesini *et al.*, 2006) that allows the relative quantification of ceramide, using fluorescence-activated cell sorting (FACS). The diacylglycerol kinase (DGK) assay (Bielawska, Perry *et al.*, 2001; Takahashi, Inanami *et al.*, 2006) is another way to measure the endogenous levels of ceramide that could also be used, but more difficult to implement as part of the method consists in a thin layer chromatography (TLC).

Another point that would be interesting to consider in the continuation of the project is the efficiency of the inhibition of the ceramide synthesis through the different pathways by the inhibitor concentration used. A measurement of the activity of the key enzymes in ceramide generation could be assessed, in the presence or absence of the inhibitors as described, for instance, by Takeda and collaborators (Takeda, Tashima *et al.*, 1999). It would give us answers about the level of inhibition of the principal ceramide generation pathways. However to apply such activity assays it would be necessary to lyse cells to obtain the enzymes, and use radioactive substrates to monitor the enzyme reaction with a scintillation counter. The protein concentration could be measured using a normal protein assay kit (Takeda, Tashima *et al.*, 1999).

Trace ceramide metabolism would also provide interesting complementary information, as it would help to confirm if and how acetate triggers ceramide synthesis. To achieve that, ceramide synthesis would be followed using radioactive compounds that would enter the sphingolipid metabolism (Luberto, Hassler *et al.*, 2002; Litvak, Bilchik *et al.*, 2003). Briefly, the method would consist in the addition of a radioactive substrate (the first in the pathway which would be studied) to the culture medium (inhibitors could also be added right after, depending on the conditions wanted). After that cells would be collected and lipids extracted using the Bligh and Dryer method (Bligh and Dyer, 1959), for example. The resultant organic phase would be used for ceramide determination, obtained through TLC, used for separation of extracted lipids. The ceramide band formed would be recognized by comparison with a standard and radioactivity measured using a scintillation counter (Luberto, Hassler *et al.*, 2002).

To summarize, our results demonstrated that ceramide synthesis pathways do not appear to be involved in the mechanisms related to acetate-induced cell death. Viability assays performed are not sufficient on their own to indisputably confirm such suppositions, but they certainly are an indicator to take into consideration. In this perspective, complementing studies have been made by our research group indicating that acetate stops proliferation and triggers apoptosis on its own (using different assays: BrDU incorporation, sulforhodamine B (SRB), TUNEL, caspase 3 activity, AnnexinV/PI staining) and it is responsible for lysosomal membrane permeabilization (LMP), and cathepsin D release into the cytosol (Marques, Oliveira *et al.*, 2012 ; submitted).

Continuation of the investigation would help in the quest to find more reliable answers and explanations about the mechanisms hiding behind the role of ceramide in acetate-induced cell death.

7

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